Investigation of transduction mechanisms for agonist-induced eosinophil responses

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To Jill

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Contents

Abbreviations

APS	ammonium persulphate
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
Bromophenol blue	3',3'',5',5''-tetrabromophenolsulphonephthalein
BSA	bovine serum albumin
C5a	complement fragment 5a
Ca^{2+}	calcium
$[Ca^{2+}]_i$	intracellular free calcium concentration
CDG	chronic granulomatous disease
DAG	diacylgycerol
Dexamethasone	9-α-fluoro-16α-methyl-prednisolene
Dibutyryl cAMP	N^6,2'-o-dibutyryladenosine 3':5'-cyclic monophosphate
DMSO	dimethyl sulphoxide
DPI	diphenylene iodonium
ECL	enhanced chemiluminescence
ECP	eosinophil cationic protein
EDF	epidermal growth factor
EDN	eosinophil-derived neurotoxin
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethylether)N,N,N',N'-tetraacetic acid
ELAM-1	endothelial leukocyte adhesion molecule-1
EPO	eosinophil peroxidase
Erbstatin analogue	methyl 2,5-dihydroxy cinnamate
Euthatal	pentobarbitone sodium
FAD	flavin adenine dinucleotide
fMLP	N-formyl-Met-Leu-Phe
Genistein	4',5,7-trihydroxyisoflavone
H_2O_2	Hydrogen peroxide
Heparin	heparin sodium
HBSS	Hank's balanced salt solution (without Ca^{2+}/Mg^{2+})/BSA (0.1%)
HUVEC	human umbilical vein endothelial cells
Fura-2AM	{1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N' -tetraacetic acid, pentaacetoxymethyl ester}
Fluo-3AM	{1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)]-2(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid pentaacetoxymethyl ester}
ICAM-1/-2	intracellular adhesion molecule-1/-2
IL	interleukin
$Ins(1,4,5)P_3$	inositol 1,4,5-trisphosphate
LTB_4	leukotriene B_4

LTC_4	leukotriene C_4
LPS	lipopolysaccharide
Macrodex	6% w/v dextran 70 in normal saline
Mg^{2+}	magnesium
MBP	major basic protein
MCP-1/-3	monocyte chemotactic peptide-1/-3
MIP-1α/β	macrophage inflammatory protein 1α/β
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NAP	neutrophil activating peptide
O_2^-	superoxide
o-Phospho-DL-serine	DL-2-amino-3-hydroxypropanic acid 3-phosphate
o-Phospho-DL-threonine	DL-2-amino-3-hydroxybutanoic acid 3-phosphate
o-Phospho-DL-tyrosine	DL-3-[4-hyydroxyphenyl]alanine 4'-phosphate
PAGE	polyacrlamide gel electrophoresis
PAF	platelet activating factor (1-o-hexadecyl-2-acetyl-sn-glycerol-3-phosphorylcholine)
PDGF	platelet-derived growth factor
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
PGI_2	prostacyclin I_2
phox	phagocyte oxidase
PI 3-kinase	phosphoinositide 3-kinase
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PKC	protein kinase C
PTK	protein-tyrosine kinase
Ponceau S	3-hydroxy-4-[2-sulpho-4(sulphophenylazo]-2,7-naphthalenedisulphonic acid
Protogel	30%(w/v) acrylamide & 0.8% bisacrylamide stock solution (37.5:1)
RANTES	regulated upon activation in normal T cells expressed and secreted
ROCC	receptor operated calcium channel
Scopoletin	7-hydroxy-6-methoxy-2H-1-benzopyran-2-one
SDS	lauryl sulphate (sodium dodecyl sulphate)
SOD	superoxide dismutase
TEMED	N,N,N',N'-tetramethylethylenediamine
TGFα/β_1	transforming growth factor α/β_1
TNFα/β	tumour necrosis factor-α/β
Triton X-100	octylphenoxyolyethoxyethanol
Tween 20	polyoxyethylene-sorbitan monolaurate
TXB_2	thromboxane B_2
Tyrphostin A47	(3,4-dihydroxybenzylidene)-thiocyanacetamide
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late activation antigen-4

Abstract

Guinea pig eosinophils, human eosinophils and the eosinophilic cell lines Eol-1 and Eol-3 were compared with a range of added agonists. The three functional responses measured were hydrogen peroxide production (H_2O_2), cellular adhesion and the elevation of intracellular free calcium ($[Ca^{2+}]_i$). Human recombinant C5a, PAF, MCP-3 and RANTES were able cause H_2O_2 production, cellular adhesion and $[Ca^{2+}]_i$ elevations from human eosinophils. MIP-1α was able to elevate $[Ca^{2+}]_i$, but not produce H_2O_2, whereas IL-5 had the opposite effect. C5a, LTB_4 and to a lesser extent PAF, were able to elicit H_2O_2 production from guinea pig eosinophils. In addition, all three caused cellular adhesion and $[Ca^{2+}]_i$ elevations in guinea pig eosinophils. C5a, PAF, RANTES, MCP-1 and IL-8 were able to cause $[Ca^{2+}]_i$ elevations in the human eosinophilic cell lines, but no agonist caused any significant H_2O_2 production.

Extracellular Ca^{2+} and Mg^{2+} were required for eosinophil H_2O_2 production and cellular adhesion, whereas only extracellular Ca^{2+} was required for $[Ca^{2+}]_i$ elevation. SK&F 96365, a reported blocker of receptor operated calcium channels, exposed a disparity between guinea pig and human eosinophil responses. Ro 31-8220/002, a selective protein kinase C (PKC) inhibitor, caused a concentration-dependent inhibition of H_2O_2 production and adhesion by both guinea pig and human eosinophils. Ro 31-8220/002 caused a concentration-dependent potentiation of agonist-stimulated elevations in $[Ca^{2+}]_i$ in guinea pig eosinophils. Eosinophils appear to have extensive tyrosine kinase phosphorylation, but the specific inhibitors genistein and herbimycin-A, had no significant effects on H_2O_2 production or $[Ca^{2+}]_i$ elevations. Finally, wortmannin, a proposed inhibitor of phosphoinositide 3-kinase (PI 3-kinase) concentration-dependently inhibited eosinophil H_2O_2 production. Also, wortmannin had no effect on elevations of $[Ca^{2+}]_i$ in human eosinophils stimulated with various agonists.

This thesis indicates that differences exist between guinea pig and human eosinophils and the agonists that elicit responses. However, there appears to be a dissociation of the respiratory burst mechanism and rapid elevation of free calcium in eosinophils. In addition, to date there is no cell line that reflects the characteristics of blood derived mature human eosinophils.

1 Introduction

1.1 Eosinophils

Paul Ehrleich in 1879 (1) first characterised the eosinophil leukocyte that is derived from the haematopoietic stem cell primarily in the bone marrow. The special properties of eosinophils is their capacity to bind negatively charged dyes including the brominated fluorescein compound which had been synthesised and called eosin (Eos was the goddess of dawn in greek mythology) in 1866.

Blood eosinophils obtained from healthy individuals have a diameter of 8μm and a volume of 275*fl*. The nucleus occupies approximately one fifth of the cell volume and the specific granules one fifth of the cytoplasm (2). Figure 1.1 shows an electron micrograph of an eosinophilic leukocyte.

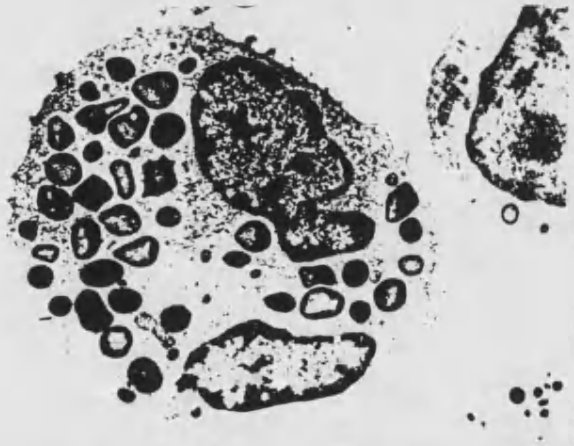


Figure 1.1 Electron micrograph of an eosinophil leukocyte. Reproduced with kind permission from *'The molecular biology of the cell'*, figure 17.27C, page 975, 2nd edition, edited by Alberts *et al* (1989), Garland Publishing Inc. courtsey of Dorothy Bainton.

Eosinophils are tissue cells as they are normally recruited to fight diseases and large numbers can be found in tissues even when blood counts are low. The number of eosinophils in the blood are a poor indication of disease due to the relationship of the number of cells that are 1) in the marrow, 2) marginating and then leaving the

blood and 3) the number present in the tissues (3). There is virtually no recirculation of eosinophils back from tissues to blood. Figure 1.2 shows a schematic representation of the eosinophil life cycle.

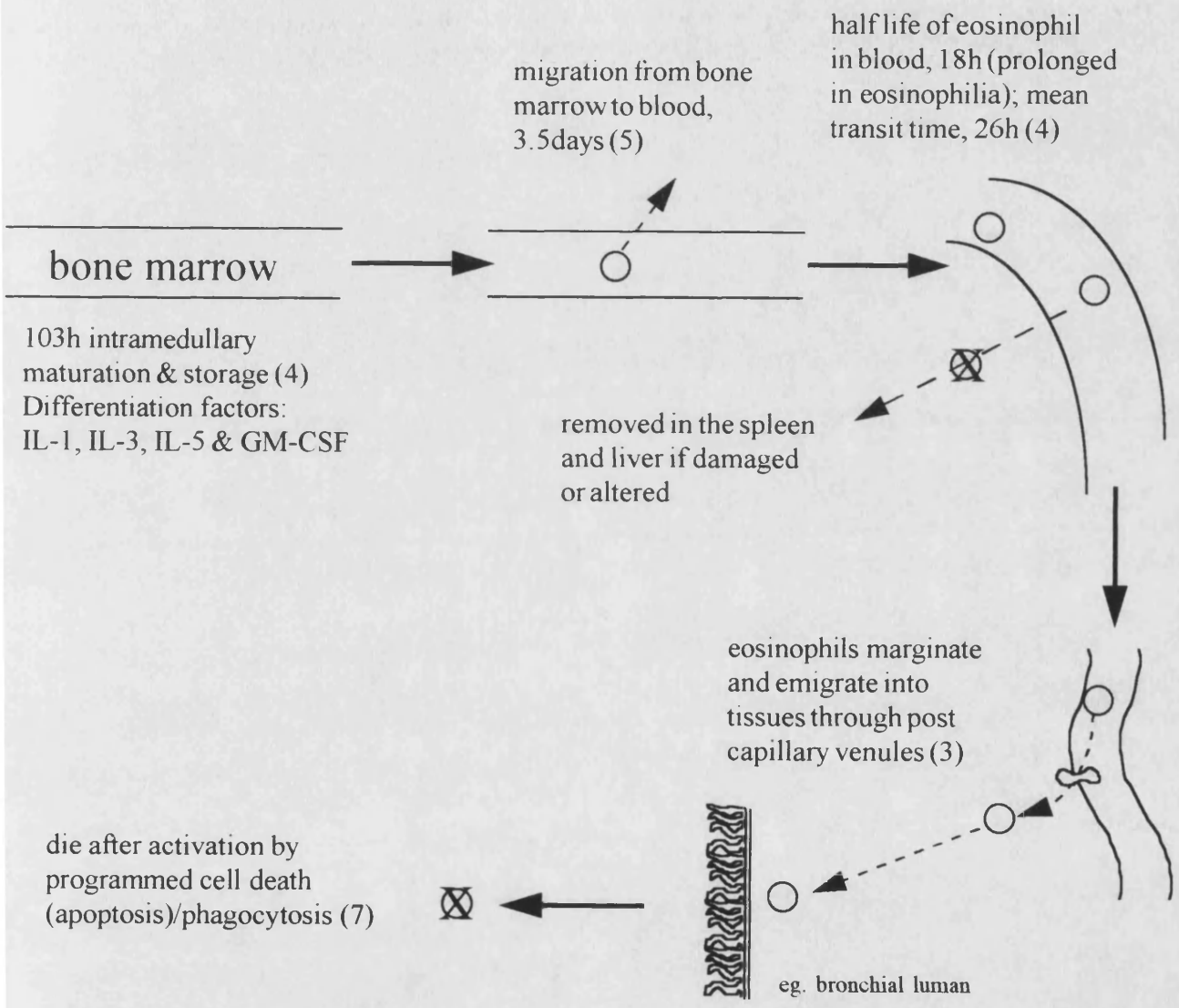


Figure 1.2 Schematic diagram of the life cycle of the eosinophil.

The nucleus of the eosinophil is bilobed but can increase to four lobes in some diseases. Four cationic proteins comprise the bulk of the protein within the distinctive eosinophil cytoplasmic granules. Major basic protein (MBP) (mw. 10kDa) is localised in the core and accounts for 55% of the granule protein. MBP is not present in other eosinophil organelles nor plasma cells, mast cells, lymphocytes or neutrophils. Eosinophil cationic protein (ECP) (mw. 21kDa), eosinophil peroxidase (EPO) (mw. 75kDa) and eosinophil derived neurotoxin (EDN) (mw. 18kDa) are all localised in the matrix of the leukocyte. Similarly acetylcholinesterase, acid glycerophosphatase, adenosine triphosphatase, α-mannosidase, arylsulphatase, catalase, β-glucuronidase, nonspecific esterase, ribonuclease, cathespin, acid and alkaline phosphatase, histaminase, phospholipase D, phospholipase β, serine pyruvate aminotransferase have also been found (8). The small granules of eosinophil contain arylsulphatase and acid phosphatase (8).

During purification of eosinophils from humans and animal models, a population of eosinophils with a lower than normal density have been isolated and have been since named light density or hypodense eosinophils. Hypodense eosinophils are connected with eosinophilia-associated diseases, parasitism and allergy and their numbers are positively correlated with the degree of eosinophilia (9). They have a peak density of 1.075-1.077 g/l as opposed to the peak density of normodense eosinophils 1.088g/l (9-11). In peripheral blood cells of normal persons less than 10% of the eosinophils are hypodense. Hypodense eosinophils are considered to be activated cells as they are 1) present in higher proportions in patients with eosinophilia, 2) appear to be more metabolically active than normodense eosinophils as they have

an increased generation of the superoxide ion and release more ECP and EDN in hypereosinophilic syndrome (12). They have an increased LTC_4 production, an increased cytotoxicity for antibody-coated targets and have more receptors for IgG_{Fc}, IgE_{Fc} and complement (11). There is controversy as to whether hypodense eosinophils originate from the bone marrow or are derived in the blood and tissues from normodense cells. Hypodense eosinophils may be partially degranulated by a prior activation step and the smaller granule size may be due to piecemeal degranulation (13).

Another property of eosinophils is that they exhibit a marked autofluoresence in comparison with other leukocytes. The excitation 380nm and 450nm and single emission of 520nm exhibited is characteristic of the flavins. Mayeno *et al* (14) have identified flavin adenine dinucleotide (FAD) as the predominant fluorophore in eosinophil granules. It is unclear whether the flavin exists as free FAD within the granules or as a prosthetic group via non-covalent attachment to granule proteins. The eosinophil granule FAD may be associated with granule oxidases and may serve as a flavin reservoir enhancing the activity of the NADPH oxidase (see section 1.4).

In addition to the recruitment of eosinophils in inflammatory processes they can affect other cells because of the mediators/cytokines they produce. Some of the cytokines they produce also cause autoactivation. Other receptors expressed include $Fc_\gamma RII$ for IgG, $Fc_\epsilon RII$ for IgE and $Fc_\alpha R$ for IgA (15-17).

1.2 Recruitment of eosinophils

Once eosinophils have been produced in the bone marrow and are circulating in the blood, they must be recruited before they can interact at the site of inflammation and exert their functional responses (see figure 1.2). First eosinophils loosely tether to the endothelial cells lining the vascular lumen. Next the cells spread then once firmly adhered undergo diapedesis. They then migrate up a chemotactic gradient through the extracellular matrix to the inflammatory focus, for example the bronchial lumen in the case of respiratory diseases.

1.2.1 Eosinophil adhesion

Adhesion is now considered to be an important stage in cellular recruitment. Adhesion proteins can determine the type of cells that infiltrate into the tissues and also can enhance the adhesiveness of activated cells. Adhesion molecules can be divided into three families: 1) the integrins,which are heterodimeric molecules that function both as cell-substratum and cell adhesion receptors, 2) the immunoglobulin superfamily, which is important in cell-cell adhesion especially during embryogenesis, wound healing and the inflammatory response and 3) the selectins, which mediate whole blood cell/endothelial cell adhesion (18).

The general mechanism of cellular recruitment is thought to be as follows: Inflammatory substances activate the circulating leukocytes and the adjacent endothelium. This activation results in changes in the adhesion molecule expression by upregulation or conformational changes with the consequence that the endothelium, the leukocytes or both become more adhesive. The leukocytes then migrate to the

vessel wall and form a loose association with the endothelium and slowly 'roll' along the surface. The cell then firmly adheres to the endothelium and diapedeses between the junctions of endothelial cells and finally makes its way through the extracellular matrix to the site of inflammation. Evidence is increasing that adhesion molecules are present on eosinophils and have an important role, though differences may exist depending whether the cells are recruited in the lung or other organs. Eosinophils express various adhesion receptors which are summarised in table 1, together with the endothelium counter receptor. These have been identified using a variety of monoclonal antibodies.

Table 1.1 Possible eosinophil adhesion receptors

Receptor	Family/Type	Counter receptor	Family/Type	Reference
VLA-4 ($\alpha_4\beta_1$)	Integrin	VCAM-1 (Fibrinogen)	Ig (matrix protein)	(19,20)
VLA-6 ($\alpha_6\beta_1$)	Integrin	(Laminin)	(matrix protein)	(21,22)
L-selectin	Selectin	Sialyated Lewis X?	(carbohydrate)	
PECAM-1	Ig	?	Integrin?	(17)
Sialyated Lewis X	(carbohydarte)	E-selectin	Selectin	(43)
CD11a/CD18 (LFA-1 $\alpha_L\beta_2$)	Integrin	ICAM-1, ICAM-2	Ig	(20,22)
CD11b/CD18 (Mac-1 $\alpha_M\beta_2$)	Integrin	ICAM-1 (C3bi, fibrinogen)	Ig (complement fragment, matrix protein)	(22,23,25)
CD11c/CD18 (p150,95 $\alpha_x\beta_2$)	Integrin	(C3bi?, fibrinogen?)	(complement fragment, matrix protein)	(22,24,25)

The integrin family (25), are transmembrane heterodimeric glycoproteins consisting of noncovalently associated α and β subunits. There are at least eleven

different α-subunits and seven different β-subunits that are associated to form at least fourteen different integrins.

The β_2 integrins are also known as leukocyte integrins because their expression is limited to white blood cells. The α-subunits (mw. 150-180kDa) have a small degree of homology with each other though they are similar in structure. All are typical glycoproteins with a N-terminal signal peptide and a relatively short cytoplasmic region. Each has a domain of three cation binding repeats that underlines the Ca^{2+} and Mg^{2+} binding requirements of the leukocyte integrins. The β_2 integrins (LFA-1, Mac-1 and p150,95) are present on all leukocytes and have a wide range of adhesion dependent functions.

There appears to be an association of integrins with cytoskeletal actin filaments via an indirect linkage involving talin, vinculin and other cytoskeletal associated molecules (26) and this binding requires the cytoplasmic domain of the β-subunit. A tyrosine phosphorylation site exists in the cytoplasmic domains of the sequences of the β_1- and β_3-subunits (18,27). Much of the work on integrins and understanding has been established with the use of specific monoclonal antibodies to the different subtypes and the rare autosomal recessive immunodeficiency disease termed Leukocyte Adhesion Deficiency (LAD). This disease is caused by a defect in the common β_2-subunit (28).

The β_1 integrins consist of receptors that bind extracellular matrix proteins. VLA-4 is the only β_1 heterodimer which has been ascribed a role in cell-cell interaction and homotypic adhesions (29). VCAM-1, the ligand that binds to VLA-4, is expressed when cytokines stimulate vascular endothelial cells, which is a pathway distinct from the CD11/CD18 interaction. This was reiterated as eosinophils have been found in

tissues from a patient with Leukocyte Dysfunction (30). VLA-4 is not expressed on neutrophils and its presence on eosinophils is likely to be highly important in eosinophil adhesion interactions.

Eosinophils have been shown *in vitro* to have increased adhesion to unstimulated endothelial cells by the cytokines IL-3 and IL-5, whereas neutrophil adhesion was unchanged. GM-CSF upregulated both eosinophil and neutrophil adhesion to unstimulated HUVEC. This could be inhibited using a monoclonal antibody to the α-chains eg. anti-Mac-1 and to a lesser extent anti-LFA-1(31). Optimal enhancement of eosinophil adhesion was between 60 and 120 minutes.

E-selectin is not constitutively expressed on endothelium but is rapidly induced *in vitro* on HUVEC by IL-1, LPS or TNFα with maximal expression at 4h. E-selectin acts as a lectin recognizing various forms of the Lewis X glycan that may be attached to membrane glycoproteins or lipids. It appears to function in the early steps of binding to endothelium as demonstrated in the neutrophil (32).

From the immunoglobulin superfamily two intracellular adhesion molecules ICAM-1 (33) and ICAM-2 (34) have been identified. These are present in low levels *in vitro* on HUVEC and expression is increased upon stimulation with TNFα, IL-1 and IFNγ (34). In addition, these inflammatory cytokines cause expression of VCAM-1, which also belongs to the immunoglobulin superfamily (35). However, VCAM-1 is only minimally expressed on unstimulated HUVEC. When stimulated the rate of expression is slower than that for E-selectins with peak expression at 24h.

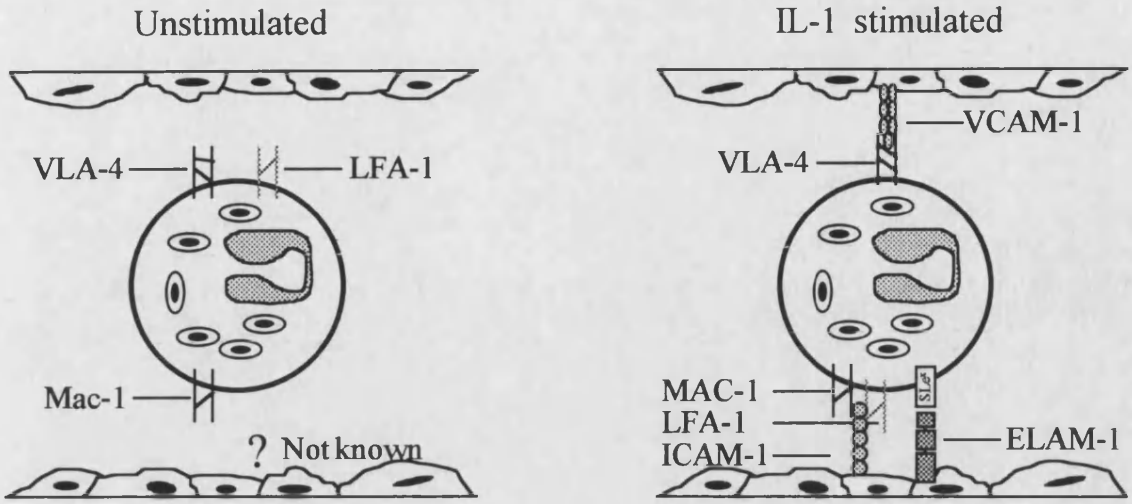


Figure 1.3 Various adhesion molecules and their counter-receptors thought to be involved in eosinophil adhesion to resting and cytokine-stimulated endothelial cells. Adapted from *'Immunopharmacology of eosinophils'* edited by Smith, H. and Cook, R.M. (1993)

Using *cos* cells transfected with cDNA for ELAM-1 or ICAM-1, eosinophils can bind to either ELAM-1 or ICAM-1 expressed in isolation, and unlike Mac-1, adhesion did not depend on eosinophils being activated by preincubation with inflammatory substances. Figure 1.3 summarises possible eosinophil adhesion interactions.

1.2.2 Eosinophil chemotaxis

Once eosinophils have adhered to the endothelium and diapedesed into the extracellular space, they move to the inflammation foci by chemotaxis. Many chemotactic agents for eosinophils have been described, but the majority are neither potent nor specific for the eosinophil.

Those that are well characterised are PAF (36), C5a (37) and LTB_4 (38). Other weak eosinophil chemotactic agonists include the cytokines IL-3 (39), IL-5 (40) and GM-CSF (39). In addition two other cytokines that have been reported to be more

potent than PAF and C5a as chemotactic agents include IL-2 (41) and lymphocyte chemoattractant factor (LCF) (42). In asthmatics numerous cells have been found to express the mRNA encoding IL-2 (43) suggesting that IL-2 may be released in an asthmatic reaction. LCF is produced from $CD8^+$ T lymphocytes in cell culture. Again activated $CD8^+$ T lymphocytes are present in airways of asthmatics following allergen challenge (43). Whether IL-2 and LCF are actually produced in the airways remains to be determined (43). Finally, RANTES (a product from T-cells), has also been found to be chemotactic to eosinophils *in vitro* (44). Chemotaxis is very different *in vitro* to the *in vivo* conditions largely due to the very controlled conditions of the Boyden chamber in which *in vitro* experiments are performed and the vast number of mediators present at the site of inflammation *in vivo*. Experiments have been undertaken to measure chemotaxis *in vivo* to C5a, PAF and LTB_4 using ^{111}Indium-labelled eosinophils in conjunction with various antagonists (45,46). These have failed to identify an agent specifically involved in the recruitment of eosinophils, suggesting the recruitment process is extremely complex.

It appears that chemoattractants such as C5a, LTB_4 and PAF encourage eosinophil transendothelial migration through upregulation of adhesion molecules and IL-2, IL-5, LCF and RANTES (47-49) aid migration through the tissue matrix and induce further cell influx. However, many of these chemotactic agents affect several cell types; eosinophil specificity may therefore arise through the adhesion molecules such as VLA-4/VCAM-1.

1.3 Eosinophil Function

Once eosinophils have reached their destination they are capable of eliciting various functional responses. They can release their cytotoxic proteins via degranulation, toxic metabolites or a range of cytokines and mediators that activate and recruit additional cells.

1.3.1 Eosinophil degranulation

As already described eosinophils contain several highly basic proteins that are extremely toxic. These are located in the secretory granules of the eosinophil leukocyte. The granule proteins include MBP, ECP, EDN, and EPO.

MBP (mw. 10kDa) is toxic to parasites (50), murine tumour cells and many mammalian cells (51). MBP induces airway constriction and hyper-responsiveness in primates (52). This effect was specific to MBP as other granule proteins had no effect. MBP also causes histamine release from basophils and rat mast cells (53) and neutralizes heparin (54). The toxicity of MBP could be inhibited by acidic poly-amino acids (55). This represents proMBP that is altered by the endoplasmic reticulum before it is sequestered in the eosinophil granule as toxic MBP.

ECP shortens coagulation time (56) and alters fibrinolysis (57). It is also very toxic to parasites (58) though its structure and molecular weight is different to MBP. Also, like MBP, ECP causes histamine release from rat mast cells. ECP is a potent neurotoxin (8) and inhibits proliferation of peripheral blood lymphocytes in cultures (60). EDN is a potent neurotoxin (59). EPO exists as either a monomer or a dimer and both have the same specific activity. EPO is just one of the eosinophil associated

enzymes. In the presence of H_2O_2 and halide it kills microorganisms (62) and tumour cells (63), initiates mast cell secretion (64), inactivates leukotreines (65) and causes histamine release from rat mast cells. Other enzymes present in the eosinophil include collagenase (66) and elastase (67).

Also present in the granules are other enzymatic proteins which include elastase, histaminase, phospholipase and arylsulphatase B. *In vitro* experiments have shown exposure of the human eosinophil to immunoglobulins or some complement products, either in the form of immune complexes or bound particles gives rise to the secretion of granule proteins. However, receptor binding is either not optimal or of a sufficient signal since there is very limited secretion unless the ligand is bound to a particle of a non-phagocytosable size. Therefore very little secretion actually takes place when the eosinophil is exposed to soluble stimuli such as PMA or calcium ionophores (68). The most potent particulate stimuli are particles coated with either sIgA or C3b (69) that release 15-25% of the content of normal human eosinophils. However, patients with eosinophilia release significantly more granule proteins. This may be due to the priming of the cell. *In vivo* experiments provide no data on selective release of any granule protein indicating that all four granule proteins are released together. Unlike ECP, EDN and EPO, MBP can be released from different sources, therefore results from *in vivo* experiments must be interpreted with care. Eosinophils in diseased tissues have shown some granules with intact cores while other granules in the same cell have lost most of their cores. This may be a sign of early activation and the beginning of degranulation (70).

1.3.2 Eosinophil superoxide release

Eosinophils have the capacity to generate and release toxic oxygen metabolites such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) which results from superoxide dismutation and the potentially cytotoxic hypohalous acids.

First the enzyme NADPH oxidase converts O_2 to its one electron product, O_2^-, as shown in equation 1.1. Concurrently, glucose is metabolised through the hexose monophosphate shunt (71) to regenerate the NADPH that was consumed by the NADPH oxidase.

$$2O_2 + NADPH \rightarrow NADP^+ + H^+ + 2O_2^- \qquad (1.1)$$

Superoxide is a free radical since it contains an unpaired electron. Radicals are very reactive and are considered to be a mediator of cytotoxicity. However, O_2^- is a precursor for the production of many, more-reactive, oxygen species. As already stated, O_2^- can undergo dismutation to form H_2O_2 and O_2 and this is facilitated *in vivo* by superoxide dismutases (72) as shown in equation 1.2.

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (1.2)$$

H_2O_2 is a relatively potent oxidant and about 80% of the H_2O_2 generated by human neutrophils arises from the dismutation of O_2^- (73). H_2O_2 can be reduced to form a hydroxyl radical, which is a highly unstable oxidizing agent. It is believed to be formed via the Fenton reaction in conjunction with the metal ion, Fe^{3+}, that is first reduced by the O_2^- anion as shown in equations 1.3 and 1.4.

$$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2 \qquad (1.3)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\bullet \qquad (1.4)$$

Finally H_2O_2 can react with different halides to form hypohalous acids. EPO is a major protein present in the eosinophilic granules and superoxide production is often associated with degranulation. Thus, EPO with a bromide ion and H_2O_2 leads to the production of hypobromous acid as shown in equation 1.5. Again this is an extremely reactive product and can either oxidize biological molecules such as bacteria, fungi, viruses and mycoplasma to kill them, react further with H_2O_2 or it can react with primary or secondary amines to form N-bromoamines, which are stable oxidizing agents (74).

$$H_2O_2 + Br^- \xrightarrow{EPO} HOBr + H_2O \qquad (1.5)$$

Superoxide anions are the least damaging of the toxic products of oxygen metabolism, spontaneous or enzyme-catalyzed dismutation to H_2O_2 generates an extracellular source of the highly reactive, membrane-perturbing hydroxyl radical. Concomitmant release of EPO in response to agonists of O_2^- production therefore establishes conditions for the formation of potentially harmful, tissue damaging products of oxygen metabolism (75).

1.3.3 Eosinophil production of mediators

As already indicated, eosinophils can elicit a wide range of mediators to aid the inflammation response. Eicosanoids are formed from the oxidative metabolism of arachidonic acid or other polyunsaturated fatty acids. Arachidonate is oxidatively metabolized via either the cyclooxygenase pathway or the 5- or 15-lipoxygenase pathways (76,78).

Human eosinophils do contain microsomal cyclooxygenase and along with terminal enzymes of this oxidative pathway, lead to the generation and release of prostaglandin (PGE_2) and thromboxane 2 (TXB_2) (34,76,79,80).

Human eosinophils also possess 5- and 15-lipoxygenase activity. In contrast to human neutrophils that release the dihydroxy acid LTB_4 as their major 5-lipoxygenase product, human eosinophils preferentially generate and secrete LTC_4 with small amounts of LTB_4 (81). There is some doubt as to whether the 15-lipoxygenase is activated in eosinophils under physiological conditions. Detectable concentrations of 15-hydroxyeicosatetraenoic acid have been measured *in vivo* in the inflamed asthmatic airway, but this may be produced via other cells. Eosinophils also possess the necessary acetyl transferase enzyme to synthesize another lipid mediator, PAF (82).

Eosinophils have the capacity to produce a number of cytokines. The first cytokine to be recognized as being formed by human eosinophils was TGFα (83). TGFα has been found within eosinophils in various neoplastic and inflammatory lesions. More recently TGFβ has also been demonstrated to be synthesized by eosinophils (84). TGFβ has been shown to promote extracellular matrix formation and could have a role in tissue fibrosis in various organs. Numerous interleukins are also

produced by eosinophils. IL-1α has the potential to serve as a co-stimulatory factor for lymphocytes during eosinophil antigen presentation (85). IL-3 (86), IL-5 (87) and GM-CSF (88) probably exert autocrine activities by activating the functional responses of mature eosinophils as well as potentially stimulating other cell types. IL-6 also has a role in acute inflammation (89). Finally, MIP-1α (90) and TNFα (90,91) are also produced from human eosinophils.

Hence eosinophils possess the ability to produce a wide and diverse collection of lipid and polypeptide mediators leading up to and during inflammatory reactions. However, the extent to which they are produced *in vivo* is uncertain due to the complexity of the immune response with various other leukocytes being present.

1.4 Cellular Signalling

Various signalling pathways in eukaryotic cells have been established. Classically, this includes G-protein receptor activation leading to diacylgycerol (DAG) and inositol 1,4,5-trisphosphate (Ins $(1,4,5)P_3$) production, resulting in protein kinase C (PKC) activation and intracellular Ca^{2+} release (see figure 1.4). However, further investigation has shown a rich diversity of mechanisms exist in the signalling cascade.

1.4.1 NADPH oxidase - structure and assembly

As already indicated the respiratory burst is an important mechanism used by phagocytes (neutrophils, monocytes and eosinophils) in host defence on ingestion of microorganisms and by soluble stimuli. The biochemical basis for the respiratory burst is not accomplished by mitochondrial respiration, but by the NADPH (nicotinamide

adenine dinucleotide phosphate - reduced form) oxidase (71). The enzyme is located in the plasma membrane with its NADPH binding site projecting into the cytosol and O_2^- molecules being released extracellularly (92). This location guarantees optimum delivery of oxidants onto phagocytosed microorganisms.

Much of the work on NADPH oxidase has been ascertained from patients with chronic granulomatous disease (CGD) in which there is a defect in the NADPH oxidase or its activating apparatus (93). NADPH oxidase complex is made up of several components that have been characterised over the last few years (see figure 1.5). Cytosolic NADPH acts as the electron donor and oxygen acts as the acceptor (see equation 1.1). The complex is dissociated in resting neutrophils and its components are located in both the membrane and the cytosol. Upon activation the complex consists of at least six components. An NADPH dehydrogenase transfers the electrons from NADPH to the haem as the prosthetic group in the oxidase system. This was thought to be a 45kDa membrane-bound flavoprotein. This was inhibited by 36% using the inhibitor diphenylene iodonium (DPI) at a 1:1 stoichiometry (94). This has since been questioned as cytochrome *b* is also said to be a target for DPI. Cytochrome *b* accepts the single electron and donates to oxygen at the external face of the plasma membrane or phagosome (95).

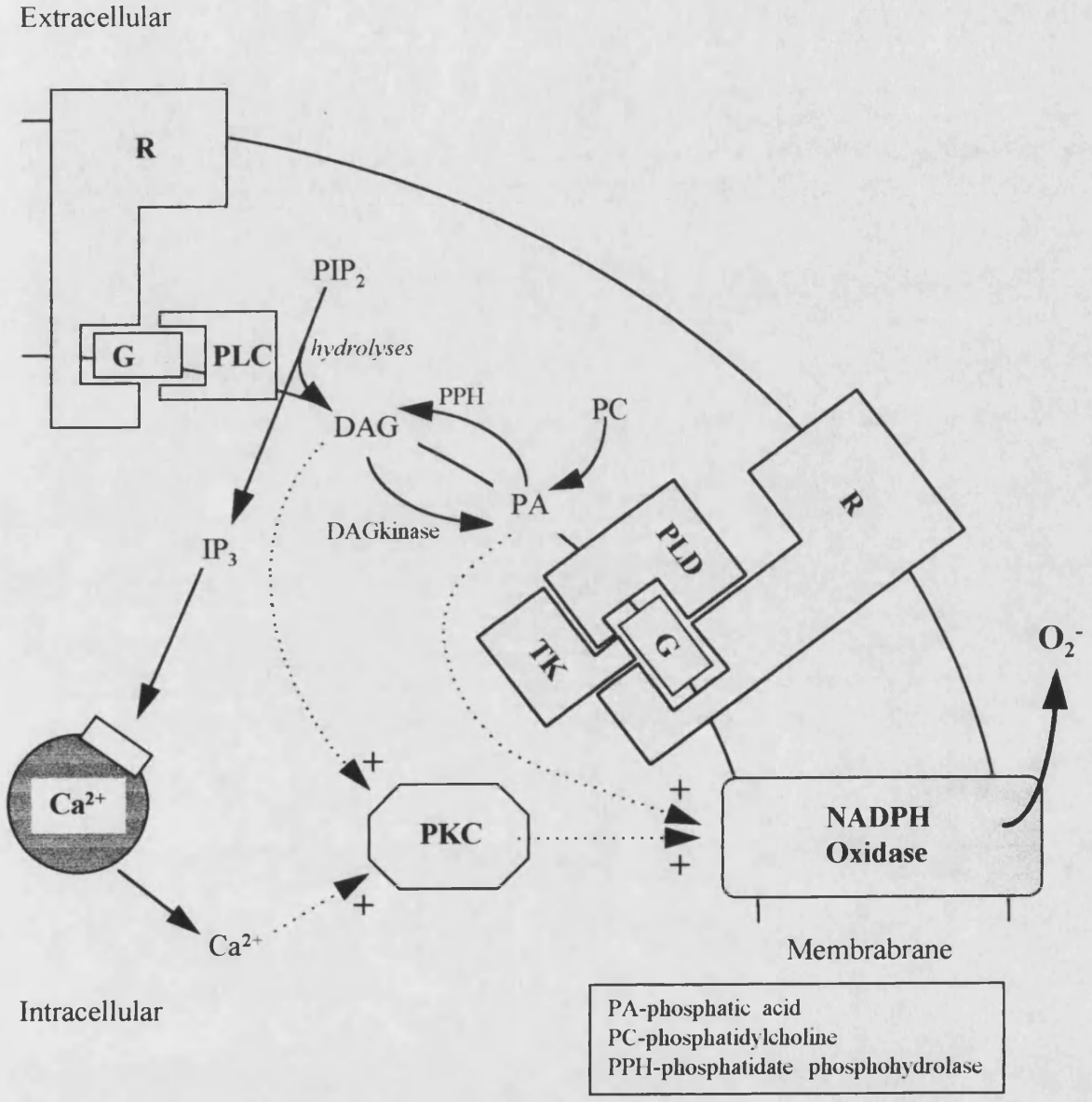


Figure 1.4 Activation of the signalling pathways via receptor coupling to PLC and PLD in neutrophils. Adapted from Garland, L.G. 1992. *FEMS Microbiol. Immunol.* **105**:229-238

The interaction of NADPH with the oxidase appears to be Mg^{2+}-dependent since O_2^- formation is inhibited by the metal-complexing agents EGTA and EDTA in proportion to their Mg^{2+} affinity. EDTA prevents the reduction of the FAD component of the oxidase by NADPH (94).

1.4.1.1 Plasma membrane components

Cytochrome *b* is the main membrane-associated component which has been characterised as having a peak of absorbance in the reduced state at 558nm and a low midpoint potential of -245mV (95). It is known as either cytochrome b_{558} or cytochrome b_{-245}. The cytochrome is a heterodimer consisting of a small α-subunit of 22-23kDa (p22^{phox}, *phox* indicates phagocyte oxidase) which carries the haem and is reported to be the terminal component of the respiratory-burst electron system (92), and a large β-subunit of 76-92kDa (gp91^{phox}) that is highly glycosylated. Other plasma membrane components include *Rap-1* a small GTP-binding protein of 21-22kDa has been found to associate with the heterodimeric cytochrome b_{558}.

1.4.1.2 Cytosolic components - p47^{phox} and p67^{phox}

Again using studies from patients with CGD who have a genetically defective oxidase that is incapable of generating significant amounts of O_2^- and H_2O_2 it was elucidated by variants of the disease, two distinct cytosolic proteins of 47-48kDa and 54-67kDa named p47^{phox} and p67^{phox} were required for normal function of the NADPH oxidase complex (96,97). p47^{phox} takes up phosphate when neutrophils are activated and lose it again when cells are returned to the resting state. Phosphorylation of isolated p47^{phox} has been observed in the presence of purified PKC suggesting p47^{phox} is a direct substrate for PKC. p47^{phox} shows a number of potential phosphorylation sites characterised by serine residues flanked by arginine residues clustered within highly basic carboxyl-terminal of the protein (98).

Other cytosolic components involved in the NADPH oxidase have been

identified as members of a *ras*-related superfamily. *Rac-1* and *rac-2* that have a molecular weight of 22kDa are GTP-binding proteins. This implies that *rac* regulatory proteins such as GTPase-activating protein, GDP dissociation inhibitors and GDP/GTP dissociation stimulators will also be important in controlling the activity of the NADPH oxidase system.

Similar results on the NADPH oxidase complex have been obtained using HL-60 cells differentiated to granulocytic cells (99), where upon differentiation towards eosinophils leads to increase in cytochrome b_{558} in the membrane and thus superoxide generation. Results indicate the amount of oxidase activity is dependent on the amount of cytochrome b_{558} in the membrane and the level of the translocated cytosolic factors on the membrane. A schematic diagram of the NADPH oxidase is shown in figure 1.5.

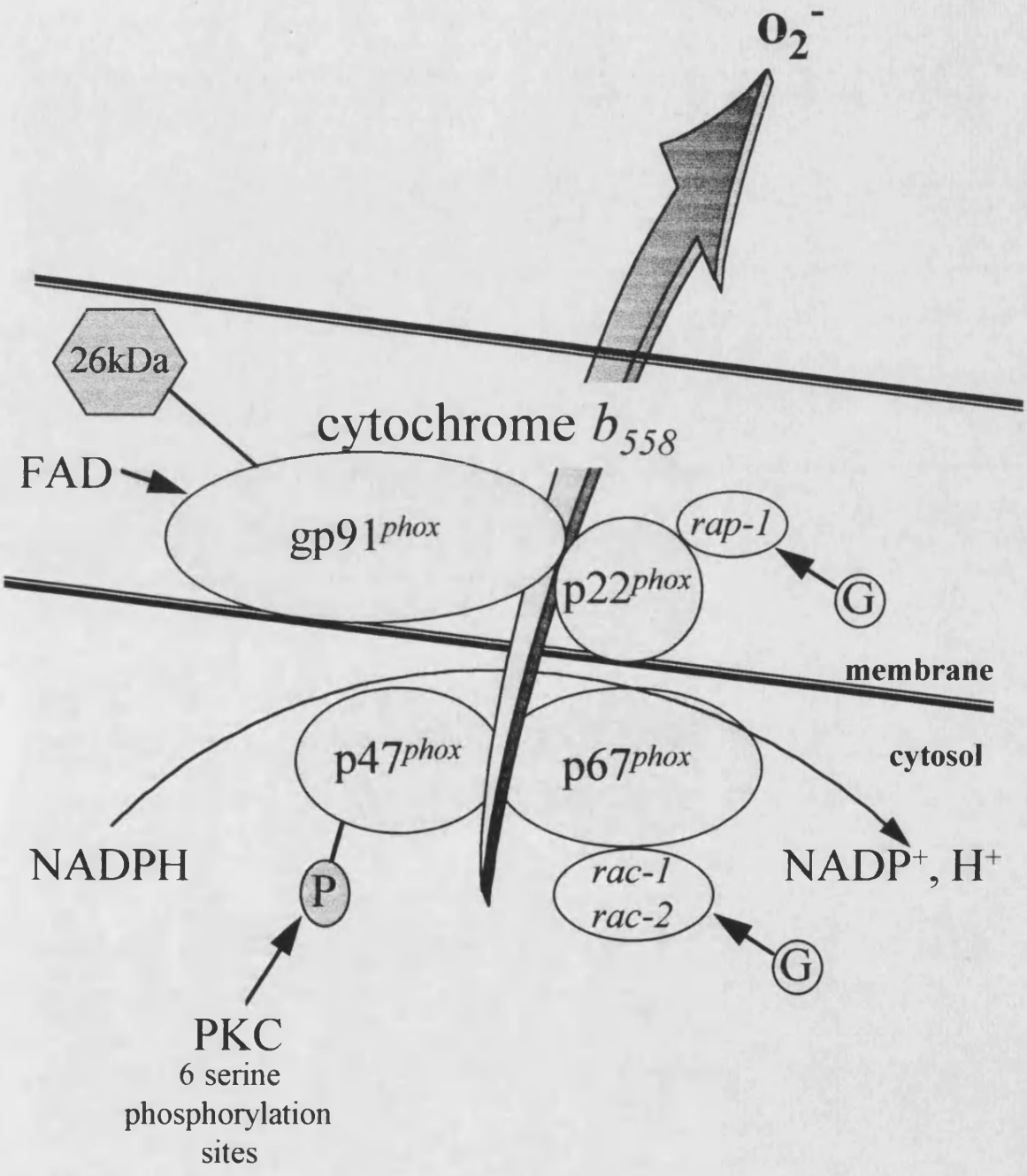


Figure 1.5 Schematic diagram of the NADPH oxidase complex in neutrophils

1.4.2 Role of intracellular and extracellular cations on cellular function

Cells must regulate ion concentrations, which means transporting specific ions into or out of the cell. The selective permeability of biological membranes to simple ions creates large differences in the ionic composition of the cell interior compared to the extracellular fluid. For example the total intracellular calcium concentration in a typical mammalian cell for calium and magnesium is 1-2mM (0.1μM is free) and 30mM respectively whereas the extracellular concentrations for these ions are 2.5-5mM and 1-2mM (100). This enables cell membranes to store potential energy in the form of ion gradients. Transmembrane ion gradients are used to make ATP, to drive various transport processes and to convey electrical signals (100).

Calcium has a very dynamic role in cell signalling both inside and outside the cell and is present in all eukaryotic cells. An increase in $[Ca^{2+}]_i$ can trigger contraction, cellular proliferation, secretion, metabolic adjustments and changes in gene expression. Two basic mechanisms by which the cell may increase its $[Ca^{2+}]_i$ exist. Firstly via release of Ca^{2+} from its intracellular store such as the endoplasmic reticulum (ER) or secondly via the transport of ions across the lipid bilayer (which is achieved by specific transmembrane proteins) either using voltage operated (VOCC) and/or receptor operated calcium ion channels (ROCC). The Ca^{2+} ATPase in internal membranes or the Ca^{2+} ATPase and Na^{2+}/Ca^{2+} exchanger in the plasma membrane terminate the Ca^{2+} signal by moving Ca^{2+} into the internal stores or the extracellular fluid respectively (101). In non-muscle cells, a Ca^{2+}-storage organelle consists of three components. 1) a Ca^{2+}pump (slow-type Ca^{2+}-ATPase; mw. 100kDa); 2) a Ca^{2+}-storage protein (eg. calreticulin and possibly endoplasmin) which acts as a Ca^{2+} buffer

in the lumen of the Ca^{2+}-storage organelles. They reduce the lumen/cytosol Ca^{2+} gradient and diminish the risk of precipitation. Also they may be actively involved in the regulation of Ca^{2+} release; and 3) a Ca^{2+}-release channel known as the $Ins(1,4,5)P_3$-sensitive Ca^{2+} release channel (the $Ins(1,4,5)P_3$ receptor) which releases Ca^{2+} during cellular activation (102). The changes in the calcium concentrations can be accurately measured using fluorescent dyes such as fura-2 (103) and fluo-3 (104) to evaluate the physiological role of cytosolic free Ca^{2+}.

$[Ca^{2+}]_i$ has been shown to change in eosinophils during chemotaxis. Experiments have shown that cells moving in one direction had a high level of $[Ca^{2+}]_i$ at the rear of the cell and low levels of $[Ca^{2+}]_i$ at the front of the cell suggesting a role in organization and local activity of cytoskeletal proteins (105) however another group has shown eosinophils depleted of Ca^{2+} appeared to still undergo shape change (106).

Evidence also suggests a role exists for $[Ca^{2+}]_i$ in O_2^- formation, as blocking $[Ca^{2+}]_i$ mobilization or depleting the $[Ca^{2+}]_i$ prevents O_2^- generation from being produced (107). However, this rise in $[Ca^{2+}]_i$ that precedes O_2^- generation in neutrophils is not sufficient to activate the respiratory burst unless the cells have been previously primed (108-110).

The first stage in eosinophil recruitment is the adhesion to the endothelial cells via various selectins and integrins. The amino acid sequence of the integrin α_M (CD11b) derived from cDNA predicts three divalent cation binding sites (111) and this requirement for Ca^{2+} and Mg^{2+} probably reflects stabilization of a conformation of the molecule necessary for ligand binding. Removal of extracellular Ca^{2+} in neutrophils affects the role of Mac-1 with regard to phagocytosis but not adhesion (112). In

neutrophils, substitution of extracellular Ca^{2+} and Mg^{2+} with Mn^{2+} increased their adhesion, as Mn^{2+} alters the affinity of integrins for their ligands (113). Further investigations to study the role of divalent cations have shown neutrophil adhesion to depend on magnesium but not calcium (114). The role of divalent cations in functional responses has also been partly characterised in eosinophils.

Guinea pig eosinophils have been shown to require extracellular Mg^{2+} for PAF induced O_2^- production (107). Indeed as already noted in section 1.4.1 Mg^{2+} is involved in the NADPH oxidase complex (situated in the plasma membrane) (94) possibly at the level of the G-protein (115).

The external cations are also important in producing a localised signals. For example, to maintain a sustained elevation of $[Ca^{2+}]_i$, replenish $[Ca^{2+}]_i$ extruded from the cell or regulate $[Ca^{2+}]_i$ oscillations (116). Mn^{2+} can be used as an indicator of divalent cationic influx as fura-2 has a high affinity for $Mn^{2+}$and its fluorescence is quenched by Mn^{2+}. Two general classes of agents have been employed to block voltage-dependent Ca^{2+} entry. These include a diverse range of organic compounds for example nifedipine, diltiazem and verapamil. These were identified for their potent pharmacological actions rather than specific antagonists of L-type VOCC's. Also known to block Ca^{2+} currents are the divalent and trivalent cations including Ni^{2+}, Co^{2+}, Cd^{2+} and La^{3+}. These inorganic ions also block ROCC's (117). Another group of compounds, the imidazoles, are also potent inhibitors of divalent calcium uptake. These include econazole, miconazole and SK&F 96365 (120). The selectivity of these compounds for ROCC's over internal release does not translate to a selectivity between ROCC's and VOCC's (117). Influx of Ca^{2+} has been detected in platelets (119),

lymphocytes (120) and neutrophils, where experiments show agonist-induced Ca^{2+} influx is secondary to the emptying of the intracellular stores which in turn activates the plasma-membrane Ca^{2+} channels by a mechanism involving microsomal *P*-450, (121).

1.4.4 Protein kinases

Protein phosphorylation of a selected protein either increases or decreases its functional activity. A protein kinase catalyses the transfer of phosphate from a nucleotide (usually ATP) to a substrate protein. The activity of this substrate protein is thus modulated by a conformational change effected by the highly charged phosphate. The protein kinases that catalyse this reaction recognize the substrate as a specific protein or proteins and transfer the phosphate to the hydroxyl group of the serine, threonine or tyrosine residues.

1.4.4.1 Protein kinase C

Protein kinase C (PKC) acts by phosphorylating selected free hydroxyl groups on the amino acid side chain of either serine or threonine residues. The regulatory enzyme, PKC has been established to have a wide variety of roles on cellular processes. These include effects in the endocrine systems, exocrine systems, nervous systems, muscular systems, inflammation and immune systems and in metabolic systems ranging from signal transduction to tumour promotion (122). PKC is activated by the receptor-mediated hydrolysis of inositol phospholipids, and relays information in the form of a variety of extracellular signals across the membrane to regulate many

Ca^{2+} dependent processes. Frequently a positive signal is followed by a negative feedback regulation. PKC was identified as a proteolytically activated protein kinase in 1977 and is ubiquitous in tissues and organs. PKC requires Ca^{2+} and phospholipid for its activation, particularly phosphatidyl serine (124). DAG dramatically increases the affinity of this enzyme for Ca^{2+} and subsequently activates the enzyme without a net increase in the Ca^{2+} concentration (123). The activation of PKC is thought to be biochemically dependent on Ca^{2+}, but under some conditions physiologically independent of Ca^{2+}. As a consequence, phorbol esters have been widely used to try to establish the role of PKC in signal transduction. PKC has been studied extensively and ten different isoforms of PKC have been described. These include the conventional PKCs (cPKC) -α, -β_I, -β_{II} and -γ which are Ca^{2+}-dependent forms, the novel PKCs (nPKC) -δ, -ϵ, -η, and -θ which are Ca^{2+}-independent forms, and the atypical PKCs (aPKC) -ζ, and -λ which are not activated by DAG or phorbol esters (125-127a).

In human neutrophils, protein kinase C translocates from the cytosol to the plasma membrane upon stimilation (127). This process is Ca^{2+}-dependent and reversible. It is not known whether the kinase is truly cytosolic or rather localized in the periplasmic space in loose connection with the membrane (128). There are few studies of the different isoenzymes of PKC in eosinophils, but in neutrophils the α-, β- and ζ- but not γ-isotypes have been revealed. The most abundent isoenzyme in neutrophils appears to be PKC-β which does translocate to the plasma membrane in the presence of Ca^{2+} (129). Several reports have implicated a role for PKC in the respiratory burst in neutrophils (130,131) and in eosinophils (75). The cytosolic

component of the NADPH oxidase, $p47^{phox}$ (see figure 1.5), contains several recognition sites for PKC and becomes rapidly phosphorylated after receptor activation or phorbol esters (98,132). Ca^{2+}-independent PKC isoenzymes in macrophages may also have a role in the activation of the NADPH oxidase (133).

1.4.4.2 PKC Inhibitors

To investigate the role of PKC in cells, various inhibitors are available. Classically, chlorpromazine, dibucaine and other phospholipid-interacting drugs including polymyxin B were used. These inhibited PKC by competing with the phospholipid with relatively low potency (134). Isoquinoline sulphonamides including H-7 and H-9 are more specific inhibitors of PKC with an K_i of 6μM and 18μM respectively (135). These act by competing with ATP for its binding site on PKC (136). Another PKC inhibitor trifluoperzine dihydrochloride, has an IC_{50} value 7-10μM in guinea pig eosinophils (75). To identify PKC in any mechanism inhibitors do not only have to be potent at low concentrations, but selective as well, especially if inhibition of PKC might represent a target mechanism of therapeutic agents. Staurosporine, a microbial alkaloid, inhibits the proteolytically generated catalytic domain of PKC (137). Other members of the family include K252a (138) and UCN01 (139). Staurosporine and its related compounds were found to be potent inhibitors of PKC with an IC_{50} value of 2-5nM (140). Because they inhibited by competing with ATP for the ATP co-substrate site common to all protein kinases, they had limited selectivity for PKC (139). Since the use of staurosporine was introduced, other PKC inhibitors have been produced. Calphostin C inhibits PKC with an IC_{50} value of 50nM,

but does not inhibit protein kinase A or the protein-tyrosine kinase (PTK) of $pp60^{v\text{-}src}$ even at 50μM (140,141). In addition, calphostin C does not inhibit the catalytic domain of PKC but appears to interact with the regulatory domain.

A series of potent selective inhibitors of PKC based on the structure of staurosporine have been produced. Though competing with ATP for the co-substrate site of PKC, the structural modifications of the compounds have demonstrated a selectivity for PKC over both protein kinase A and Ca^{2+}/calmodulin dependent kinase (142).

1.4.5 Tyrosine phosphorylation

The vast majority of phosphorylations occur on serine. The discovery that the transforming gene of Rous sarcoma virus (*src*) encodes a protein kinase specific for tyrosine (143), initiated a whole new field of investigation to isolate other PTKs and their substrates (144). The PTKs can be divided into two groups, the transmembrane receptor family (including growth factors such as EGF and PDGF) and the cytosolic non-receptor family (including oncogene products such as $pp60^{v\text{-}src}$).

Phosphotyrosine can be detected using several techniques. These include: (1) radiolabelling proteins with ^{32}P (either [^{32}P]ATP *in vitro* or with $^{32}PO_4$ *in vivo*). The proteins are purified and subjected to partial acid hydrolysis, mixed with phosphoamino acid standards and resolved by one- or two-dimensional thin layer electrophorsis. Finally autoradiography allows comparisons between [^{32}P]phosphoamino acid with the known standards (143); (2) Phosphotyrosine is known to be relatively resistant to the base hydrolysis compared to phosphothreonine and phosphoserine and this property has

been exploited to analyse p-Tyr-containing proteins by SDS-PAGE. The gel is then soaked in base (KOH) and subjected to autoradiography (145); and (3) Anti-phosphotyrosine antibodies have been used in conjunction with immunoblotting.

1.4.5.1 Possible roles of tyrosine kinase in cellular signalling

One hypothesis for the mechanism of action of the PTKs, is the transmission of a signal to another class of proteins - the serine/threonine kinases. Early studies on the effects of PTK activation noted a parallel activation of serine/threonine kinases. Several such tyrosine-phosphorylated kinases have now been identified. These include the CDC2 protein (146), MAP kinase (147) and possibly *raf* kinase (148).

Several proteins have been shown to possess an amino acid sequence similar to already defined regions of the non-receptor PTK family known as SH2 and SH3 domains (SH = *src* homology regions). The SH2 domains are known to mediate direct interaction between the SH2-containing protein and phosphotyrosine, with some specificity observed in the amino acid sequence surrounding the phosphorylated tyrosine. One model for the mechanism of action of growth factor receptors that emerges is that upon ligand binding the PTK domain is activated or inactivated leading to autophoshorylation and association with SH2-containing proteins. The SH2-containing proteins then become tyrosine phosphorylated and activated in their own signal transduction pathway. This model suggests that the PTK substrate specificity resides at a site distant from the actual tyrosine phosphorylation site (144). These SH2 proteins include phospholipase C_γ (149), GTPase activating protein (144), and phosphatidylinositol 3-kinase (PI 3-kinase) (150).

Several membrane proteins involved with communication are also tyrosine phosphorylated. These include connexin-43 (151), the nicotinic acetylcholine receptor (152), the T-cell antigen receptor (153), caveolin (154), coated pits (155) and cAMP phosphodiesterase (156).

Tyrosine phosphorylation also occurs in cytoskeletal proteins including the focal contact proteins such as vinculin (157), paxillin (158), tensin (159), talin (160) and integrins (161) and other cytoskeletal tyrosine phosphorylated proteins such as annexin I (162), annexin II (163) and ezrin (164).

Finally there are a few other proteins that are tyrosine phosphorylated which do not belong to any particular group but may be just as important (144).

1.4.5.2 Tyrosine phosphorylation and effect on neutrophil function

No protein tyrosine phosphorylation had been established in eosinophils at the start of this project. The typical amount of tyrosine phosphorylated proteins present in well characterised cells is no more than 0.5-2% of the total protein content (eg. platelets (210)), thus the elucidation of the role that PTKs play in eosinophils is impeded by the low number of eosinophils that are available.

A rapid increase in the phosphotyrosine content of several proteins has been observed in neutrophils after stimulation with chemotactic agonists (77,165-167), but the mechanism of tyrosine kinase activation in these cells is still poorly understood. It is also apparent that the seven transmembrane-segment receptors lack tyrosine kinase domains and there is no indication that they associate with PTKs (168). A member of the *src* family $p55^{c\text{-}fgr}$ is expressed in neutrophils. It is translocated on activation from

the granules to the plasma membrane and may be involved in agonist-induced exocytosis (169). The cytosolic components of the NADPH oxidase, $p47^{phox}$ and $p67^{phox}$ contain the *src* homology domains SH2 and SH3 (which are known to mediate the phosphotyrosine-dependent association of cytosolic and membrane proteins) (98,170), however, phosphoamino acid analysis reveals no tyrosine phosphorylation of the components of the NADPH oxidase, suggesting that its assembly and activation do not depend on PTKs (171).

1.4.5.3 Tyrosine kinase inhibitors

As already noted, protein-tyrosine phosphorylation has the potential to initiate many signal transduction mechanisms, therefore as with PKC, potent and specific inhibitors of TK may provide useful means to identify the role of tyrosine phosphorylation in a variety of cellular events.

Erbstatin is produced by *Streptomyces* MH435-hF3. Erbstatin inhibits EGF receptor-associated tyrosine kinase, but does not appear to inhibit purified rabbit brain PKC and weakly inhibits protein kinase A and phosphatidylinositol kinase. From inhibition studies, erbstatin competes with the peptide substrate. Erbstatin is easily inactivated in serum and more stable analogues have been produced. The only stable analogue to date is methyl 2,5-dihydroxy cinnamate. This analogue inhibits tyrosine kinase *in vitro* but is not expected to be active *in vivo*, because it is a polar molecule and would penetrate the cells poorly (172).

Genistein is produced by *Pseudomonas* (173). Genistein has been shown to inhibit EGF receptor and $pp60^{v\text{-}src}$ *in vitro* and the EGF receptor *in vivo*, but poorly the

activity of the serine and threonine kinases such as cAMP-dependent protein kinase (174). Genistein, unlike erbstatin is a competitive inhibitor with respect to ATP. However, genistein is noncompetitive with the phosphate acceptor as it bears no structural relationship to ATP, so inhibition may occur by genistein binding in multiple places in the reaction pathway (173). Genistein does not appear to be completely selective as it has reported to inhibit β-galactosidase (175), DNA topoisomerases II and I (176) and S6 kinase activity (177).

Herbimycin A is produced by *Streptomyces* (MH237-CF8) and was found to inactivate v-*src*, in particular $pp60^{v\text{-}src}$ *in vitro*. In addition to *src*, herbimycin A is effective against the tyrosine kinase oncogenes *yes*, *fps*, *ros*, *abl* and *erbB*, but not *ras*, *mys* and the serine/threonine kinase *raf*. The effects of herbimycin A on cAMP-dependent kinase and PKC were also negative. Herbimycin A binds to active SH group(s), which are not essential for kinase activity. However, binding of the bulky compound is thought to restrict access of the ATP molecule (178).

Tyrphostins are synthetic copolymers of amino acids which contain tyrosine residues, but are devoid of serine and threonine. They have been shown to inhibit protein-tyrosine kinases (PTKs) both *in vitro* and *in vivo* (179). They act by binding ATP and thus prevent the activation of TKs (competitive inhibitor). Depending on their structure, tyrphostins can have either a broad or restricited specificity for the Tks.

1.4.6 Role of PI 3-kinase in signalling pathways

As indicated in section 1.4.5.1, tyrosine kinases can activate various pathways, each leading to highly specific responses. PI 3-kinase is involved in just one of these

pathways. Phosphatidylinositol is a unique phospholipid that can be modified by phosphorylation at any of the five hydroxyls on the inositol ring. Phosphorylation at different positions on the inositol ring provides a means for generating a wide variety of second messenger signalling molecules (180). PI 3-kinase is a heterodimer with an 85kDa regulatory subunit, which binds to tyrosine phosphorylated proteins via either of its two SH2 domains, and a 110kDa catalytic subunit, with dual specificity possessing both lipid and serine kinase activities.

PI 3-kinase, acts on phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate to produce phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate respectively (181).

PI 3-kinase activity has been found to be associated with PTK activity more frequently than any other intracellular signalling molecule and thus PI 3-kinase is important for intracellular signals initiated by PTKs.

Phosphatidylinositol 4,5-bisphosphate is a substrate for phospholipase C_γ leading to the production of the second messenger signalling molecules inositol 1,4,5-trisphosphate and DAG. However this does not appear to be the case for phosphatidylinositol 3,4,5-trisphosphate which maybe a second messenger itself. A possible target *in vitro* for phosphatidylinositol 3,4,5-trisphosphate is the Ca^{2+}- and phorbol ester-independent PKC-ζ (182).

Wortmannin, a fungal metabolite inhibits many cellular functions at micromolar concentrations. However, at low nanomolar concentrations wortmannin specifically inhibits PI 3-kinase by binding to the catalytic subunit. It has been shown to

successfully inhibit the respiratory burst in neutrophils (183), but not eosinophils.

1.5 Eosinophilic cell lines

As already indicated, the number of eosinophils present in normal subjects is low (approximately 2-5%). The number can be greatly increased in allergic or parasitic patients though these cells are more likely to be primed or activated. Therefore, obtaining large quantities of normal cells is very difficult. The advent of eosinophil cell lines has produced an alternate source of human eosinophils. These not only allow the study of functional responses to added agonists, but yield information on eosinophil differentiation and maturation. The HL-60 promyelocytic leukaemia line can be differentiated to the eosinophil subline in alkaline medium and subsequently cultured in the presence of sodium butyrate (184). A more specific cell line (Eol) has since been established.

1.5.1 Eol-1 and Eol-3 eosinophilic cell lines

Satio *et al* (185) established the first eosinophilic leukaemia cell line from the peripheral blood of a 33-year old French man with Philadelphia chromosome-negative eosinophilic leukaemia. Clinical examination in 1978 showed no abnormal findings except hypereosinophilia with 55% of the white blood cells ($14.8x10^3$cells/μl) being eosinophils. In 1984 the white blood cell count rose to $16.9x10^4$cells/μl with eosinophils at 43% and blasts 54%. Peripheral blood was obtained and the blast-rich population was purified and cultured in supplemented media. The patient was then treated with chemotherapy, unfortunately no remission could be obtained and the

patient died. Three clones with eosinophilic features were observed out of the six clones obtained and designated Eol-1, Eol-2 and Eol-3. Most of the Eol cells had a blastic appearance without cytoplasmic granules and were morphologically similar to myeloblasts or monoblasts. However a small percentage (2%) contained granules similar to eosinophilic granules. Eol-1 and Eol-3 appeared to be the dominant clones during evolution and when differentiated under various conditions matured into eosinophils. HL-60 cells however have the ability to mature to either eosinophils or neutrophils depending on the extracellular conditions. It thus seems that the Eol blasts are committed to the eosinophil lineage.

The most common maturation agent used with Eols is some form of butyrate. For example Saito *et al* used butyric acid (0.5mM) on Eol-1s for 4-7 days (186). In addition soluble factors are also thought to promote eosinophil maturation such as a derived factor from the human adult T-cell leukemia line HIL-3 or G-CSF/TNFα(187) and TNFα/IFNγ (188). IL-3, GM-CSF and IL-5 with butyric acid were able to enhance eosinophilic differentiation (186).

1.5.2 Advantages and disadvantages of the eosinophilic cell lines

The Eol-1 and Eol-3 eosinophilic cell lines are derived from a human source and if some fully matured cells can be obtained, very accurate *in vitro* experiments can be performed to elucidate structural and functional information. The cells can be grown very rapidly to produce large numbers allowing greater experimentation. However, the eosinophilic cell lines very rarely differentiated to mature eosinophil leukocytes. They are usually still someway removed from the final state of the cell.

This implies receptors may not be fully expressed and the molecular mechanisms may not be fully operational. In addition down-regulation or shedding of some receptors may not have occurred, giving the cell properties that would not be present in the matured cell. No *in vivo* experiments are possible.

Various animal models of eosinophilia exist. These include mouse, rat, guinea pig, rabbit, dog, sheep and monkey. These have been used to establish eosinopoiesis, tissue distribution of the eosinophils and the effects eosinophils have at the site of inflammation. Like the cell lines, problems exist with all the animal models. They have a degree of variation between species including the number of eosinophils in normals, the proportions of the cellular proteins they contain, the different mediators to which they react and mediators that are consequently produced. Eosinophils that are elicited in animals for *in vitro* experiments may already be preactivated. Hence though the cell lines are not ideal, they will help further the understanding of the eosinophil.

1.6 Selection of assays

To analyse the different functions of eosinophils, three key assays were adapted, namely the hydrogen peroxide assay, rose bengal adhesion assay, and measurement of intracellular free calcium elevation.

Many documented studies measure the superoxide produced from granulocytes (in suspension) via a spectrophotometric assay as the superoxide dismutates (SOD) inhibitable reduction of cytochrome *c* described by Yamashita (189). The experiments described in this study were to be carried out in microtitre plates coated with an appropriate matrix protein to try and mimic *in vivo* conditions; as demonstrated in

asthma and other inflammatory processes (190-194). The H_2O_2 assay introduced by Andreae in 1955 (195), is based on the horseradish peroxidase-catalysed oxidation of fluorescent scopoletin by H_2O_2. It has been used successfully by other researchers to measure the release of H_2O_2 from human neutrophils (196) and macrophages (197). This assay was more sensitive and gave a greater window of detection than the cytochrome *c* assay. The rose bengal adhesion assay (198) was chosen as a simple way of determining the number of cells adhered to the plate. It was convenient to measure this parameter after the H_2O_2 assay rather than carrying out a separate assay just to measure adhesion. The rose bengal stain (which is taken up by the cells) was more appropriate than a product that measures protein eg. Bradford's reagent, as this would measure the protein used to coat the plate as well.

Calcium appears to be important in many of the cellular signalling pathways. To establish the extent that Ca^{2+} has in a particular process requires a method that quantitates it accurately. One of the first fluorescent dyes developed was quin-2 (199). This has many limitations. Due to the fluorescent properties of the dye, significant autofluorescence from the cells occurs causing possible biological side effects; to overcome this autofluorescence, high loadings of quin-2 are necessary, however, this significantly buffers the $[Ca^{2+}]_i$ transients. Quin-2 signals calcium by increasing its fluorescent intensity without much shift in excitation and emission, this gives inaccurate results as the fluorescence is dependent on many other factors that are not quantified (103). Also, quin-2 binds other divalent cations such as Mg^{2+}. The fluorescent dye fura-2 has now been developed. This is 30-fold brighter in fluorescence, it also changes in wavelength upon Ca^{2+} binding, has a slightly lower affinity for Ca^{2+}, has

a slightly longer wavelength of excitation, and is considerably more selective for Ca^{2+}over divalent cations. In addition, as fura-2 operates with a dual excitation and single emission the loading is not a crucial factor between different cell preparations (103). Fura-2 was used for all experiments except when tyrosine kinase inhibitors were used. These interfered with the fluorescent properties and thus fluo-3 was utilised (104).

1.7 Eosinophils and their role in disease

The role of the eosinophil is implicated in a variety of respiratory diseases including asthma. Asthma is a disease characterized by the reversible obstruction of the airways or bronchi. This is accompanied by nonspecific bronchial hyperresponsiveness, which is the tendency of the bronchi in asthmatics to constrict in response to a wide variety of irritant and pharmacological stimuli. It is now widely accepted that chronic inflammation of the bronchial mucosal lining plays a fundamental role in the genesis of these clinical manifestations (200).

The asthmatic response occurs in two stages, the early and late phase. Initially, the F_c region of the IgE molecule binds with high affinity to specific receptor proteins on the surface of mast cells in tissues (and basophilic leukocytes in the blood). Bound IgE molecules in turn serve as receptors for antigen. Antigen binding to the IgE molecule then triggers the mast cell to secrete a variety of biologically active products. The early asthmatic reaction results from the release of histamine, prostaglandin D_2 (PGD_2) and leukotreine C_4 (LTC_4) from IgE-mediated bronchial mast cell degranulation. The spasmogenic effects of histamine account for much of the onset of

bronchoconstriction, while PGD_2 and LTC_4 and their respective metabolites become increasingly important in maintaining the early response for up to two hours after the initial challenge (201). Mast cells are present in higher numbers and have a greater ability to release mediators in asthmatic subjects compared to normal subjects. The mechanism of the late asthmatic reaction is unknown, but it appears to involve T-cell activation and the selective recruitment of the eosinophil leukocytes. Patients who have a biphasic asthmatic response following allergen challenge, seem to have a more serious type of bronchial asthma (201). Therefore, the late asthmatic reaction may induce an increase in bronchial hyperreactivity.

1.7.1 Role of eosinophils in asthma - clinical evidence

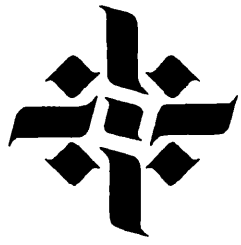
Initially, the presence of eosinophils were considered as modifiers of inflammation, but the fact that they are: (1) present in increased numbers in the circulation (9), bronchial secretions (202) and the lung parenchyma itself; (2) capable of generating substances that can cause injury to the lung and are directly toxic; (3) increased quantities of these toxic mediators are detected in the lung, eg. observed as Charcot-Leydon crystals that represent the crystaloid form of the enzyme lysolecithase from degenerated eosinophils (203), and (4) reducing the numbers of the eosinophils leads to the improvement of the clinical symptoms of asthma (204), the eosinophil is now considered to be a proinflammatory cell.

The understanding of the role the eosinophil plays in asthma and inflammatory diseases has increased rapidly. In addition, the understanding of signalling mechanisms in different cell types has moved on immensely. Various new approaches are currently

under investigation ranging from selective phosphodiesterase isoenzyme inhibitors to 5-lipoxygenase inhibitors, specific PAF and leukotriene antagonists to more specific muscarinic receptor antagonists. The pursuit of a drug that is easily administered to the site of action, highly specific, non-toxic and which reverses existing symptoms/ conditions is the ultimate goal.

1.8 Aims of this project

The aim of this study was to characterise the relationship between activation of transduction mechanisms and functional responses in eosinophils. Specifically the functional responses investigated were H_2O_2 production, cellular adhesion and elevations in free calcium in guinea pig eosinophils, human eosinophils and the eosinophilic cell lines Eol-1/Eol-3 induced by soluble stimuli implicated in the allergic response. The effect of the extracellular cationic conditions and the role of calcium influx using manganese, nickel and the ROCC blocker, SK&F 96365 were also investigated. Finally, the role of protein kinase C, protein-tyrosine kinases and PI 3-kinase were investigated using the selective inhibitors Ro 31-8220/002 (142), erbstatin, genistein, herbimycin A and tyrphostin (172,173,178,179) and wortmannin (183) respectively.



2 Materials & Methods

2.1 Materials

2-mercaptoethanol	BDH Laboratory Supplies, Lutterworth, UK
Aluminium hydroxide	BDH Laboratory Supplies, Lutterworth, UK
Ammonium persulphate	Sigma Chemical Co., Poole, UK
Antiphosphotyrosine PY20 antibody	ICN Biomedicals Ltd, Thame, UK
Antiphosphotyrosine 4G10 antibody	TCS Biologicals Ltd, Buckingham, UK
Bovine serum albumin-low endotoxin	Sigma Chemical Co., Poole, UK
Bromophenol blue	Sigma Chemical Co., Poole, UK
n-Butyric acid	Sigma Chemical Co., Poole, UK
Calcium chloride	Fisons Pharmaceuticals, Loughborough, UK
Carbon dioxide	BOC Ltd, Guilford, UK
CD16 microbeads	Eurogenetics UK, Teddington, UK
Cellophane membrane backing	Bio-Rad Laboratories, Hemel Hempstead, UK
Chromatography filter paper 3mm	Whatman Labsales Ltd, Maidstone, UK
Cyclophosphamide	Sigma Chemical Co., Poole, UK
Dexamethasone	Sigma Chemical Co., Poole, UK
Dibutyryl cAMP	Sigma Chemical Co., Poole, UK
Digitonin	BDH Laboratory Supplies, Lutterworth, UK
DMSO	Sigma Chemical Co., Poole, UK
Dunkin-Hartley Guinea pigs	University of Bath, UK
ECL reagent	Amersham International Plc, Little Chalfont, UK
EDTA	Sigma Chemical Co., Poole, UK
EGTA	Sigma Chemical Co., Poole, UK
Endothelin-1	Gift-Bayer UK, Slough, UK
Erbstatin analogue	Calibiochem-Novabiochem Ltd, Nottingham, UK
Ethanol-SLR	Fisons Pharmaceuticals, Loughborough, UK
Euthatal	Rhône Mérieux, Dublin, Ireland
Ficoll	Pharmacia Biotech Ltd, St. Albans, UK
Filter card	Bio-Rad Laboratories, Hemel Hempstead, UK
Falcon 250ml culture flasks-vented	Fahrenheit Laboratory Supplies Ltd, Bristol, UK
Falcon 96 U-bottomed/flat-bottomed flexiplates	Fahrenheit Laboratory Supplies Ltd, Bristol, UK
Falcon Flexilids	Fahrenheit Laboratory Supplies Ltd, Bristol, UK
fMLP	Sigma Chemical Co., Poole, UK
Foetal calf serum	Gibco Life Technologies Ltd, Paisley, UK
Fungizone	Gibco Life Technologies Ltd, Paisley, UK
Fura-2AM	Molecular Probes Inc., Eugene. USA
Fluo-3AM	Molecular Probes Inc., Eugene. USA
Galacial acetic acid	Fisons Pharmaceuticals, Loughborough, UK
Gelatin	Sigma Chemical Co., Poole, UK

Genistein	Gibco Life Technologies Ltd, Paisley, UK
Gentamycin	Gibco Life Technologies Ltd, Paisley, UK
Glycerol	Fisons Pharmaceuticals, Loughborough, UK
Goat anti-mouse IgG horseradish peroxidase conjugate	Bio-Rad Laboratories, Hemel Hempstead, UK
Hanks' balanced salt solutions without phenol red	Gibco Life Technologies Ltd, Paisley, UK
Hanks' balanced salt solutions without calcium, magensium and phenol red	Gibco Life Technologies Ltd, Paisley, UK
Hebimycin A	Gibco Life Technologies Ltd, Paisley, UK
Heparin	CP Pharmaceuticals Ltd, Wrexham,UK
Horse serum	Gibco Life Technologies Ltd, Paisley, UK
Horseradish peroxidase	Sigma Chemical Co., Poole, UK
Human interleukin 5	Gift-Glaxo, Stevenage, UK
Human RANTES	Gift-Glaxo, Stevenage, UK
Human complement 5a	Sigma Chemical Co., Poole, UK
Human interleukin 8	Gift-Glaxo, Stevenage, UK
Human granulocyte macrophage-colony stimulating factor	Gift-Glaxo, Stevenage, UK
Human monocyte inflammatory protein-1α	Preprotech, London, UK
Human monocyte inflammatory protein-1β	Preprotech, London, UK
Hydrochloric acid	Fisons Pharmaceuticals, Loughborough, UK
Hyperfim-ECL	Amersham International Plc, Little Chalfont, UK
Immobilon™-P transfer membranes	Millipore (UK) Ltd, Watford, UK
Interferon-γ	Gift-Glaxo, Stevenage, UK
Laminin	Gibco Life Technologies Ltd, Paisley, UK
Leishman's stain	Sigma Chemical Co., Poole, UK
Leukotriene B_4	Gift-Bayer UK, Slough, UK
Macrodex	Vestric Hospital Service, Bristol, UK
MACS column	Eurogenetics UK, Teddington, UK
Magnesium chloride	Fisons Pharmaceuticals, Loughborough, UK
Manganese chloride	Fisons Pharmaceuticals, Loughborough, UK
Marvel Non-fat milk	Premier Brands UK Ltd, Stafford, UK
Methanol-SLR	Fisons Pharmaceuticals, Loughborough, UK
Mouse IgG-whole	Sigma Chemical Co., Poole, UK
Neutrophil activating protein-2	Gift-Glaxo, Stevenage, UK
Nickel chloride	Fisons Pharmaceuticals, Loughborough, UK
Ovalbumin	Sigma Chemical Co., Poole, UK
Penicillin-Streptomycin	Gibco Life Technologies Ltd, Paisley, UK
Percoll	Pharmacia Biotech Ltd, St. Albans, UK
Pertussis vaccine	Wellcome Research Laboratories, Bechenham,UK
Phorbyl 12-myristate 13-acetate	Sigma Chemical Co., Poole, UK

o-Phospho-DL-serine	Sigma Chemical Co., Poole, UK
o-Phospho-DL-threonine	Sigma Chemical Co., Poole, UK
o-Phospho-DL-tyrosine	Sigma Chemical Co., Poole, UK
Platelet activating factor	Bachem UK Ltd, Saffron Walden, UK
Ponceau S	Sigma Chemical Co., Poole, UK
Prestained molecular weight markers-15,000→200,000	Gibco Life Technologies Ltd, Paisley, UK
Protogel	National diagonostics, Atlanta, USA
Ro 31-8220/002	Gift-Dr Nixon, Roché, Wellingborough, UK
Rose bengal	Sigma Chemical Co., Poole, UK
RPMI 1640	Gibco Life Technologies Ltd, Paisley, UK
Sarafotoxin C	Gift-Bayer UK, Slough, UK
Saranwrap	The Dow Chemical Co., USA
Scopoletin	Sigma Chemical Co., Poole, UK
SDS	Sigma Chemical Co., Poole, UK
SK&F 96365	Gift-Bayer UK, Slough, UK
SK&F (+)96365	Gift-Bayer UK, Slough, UK
SK&F (-)96365	Gift-Bayer UK, Slough, UK
Sodium azide	Sigma Chemical Co., Poole, UK
Sodium hydroxide	Fisons Pharmaceuticals, Loughborough, UK
Sterile distilled water	Steripak, Runcorn, UK
Sterile saline (0.9%)	Steripak, Runcorn, UK
Sterile sodium bicarbonate (7.5%)	Gibco Life Technologies Ltd, Paisley, UK
Sulphuric acid	Fisons Pharmaceuticals, Loughborough, UK
TEMED	Sigma Chemical Co., Poole, UK
Terumo Insulin syringe-1ml, 27G x 1½"	Prodotto Da, 3001 Leuren, Belgium
Tris/glycine/0.1%SDS (10X)-running buffer	Sigma Chemical Co., Poole, UK
Tris/glycine (10X)-transfer buffer	Sigma Chemical Co., Poole, UK
Triton X-100	Sigma Chemical Co., Poole, UK
Trizma® Base	Sigma Chemical Co., Poole, UK
Trizma® HCL	Sigma Chemical Co., Poole, UK
Trypan blue	Sigma Chemical Co., Poole, UK
Tumour necrosis factor-α	Gift-Glaxo, Stevenage, UK
Tween-20	BDH Laboratory Supplies, Lutterworth, UK
Tyrphostin A47	Calibiochem-Novabiochem Ltd, Nottingham, UK
Wortmannin	Sigma Chemical Co., Poole, UK

2.2 Production and isolation of guinea pig eosinophils

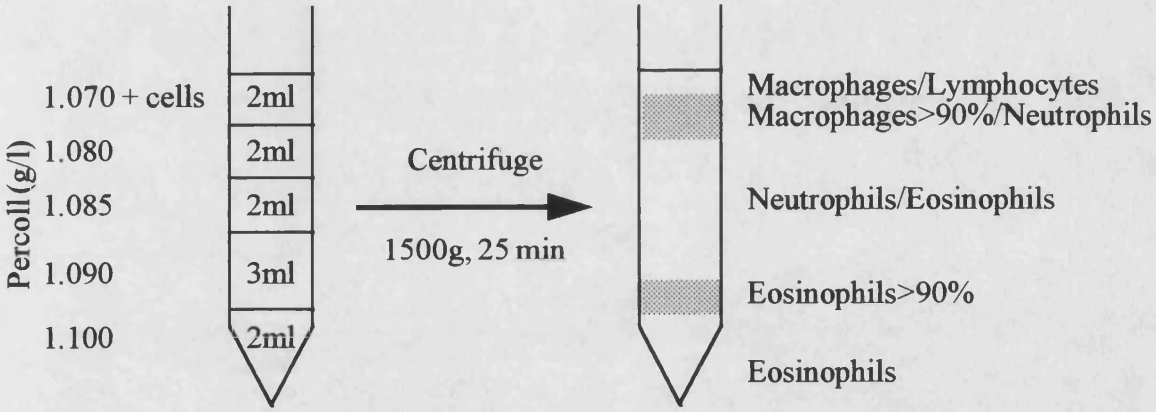
Peritoneal eosinophilia was elicited in guinea pigs as described by Litt (205). Dunkin-Hartley guinea pigs (450-600g, male and female), received a minimum of seven injections of horse serum (0.5ml, ip.) at 3-4 day intervals with the last injection a day before cell harvesting. Animals were killed by CO_2 asphyxiation. Peritoneal cells were collected by lavage with 40ml of Hanks' balanced salt solution (without Ca^{2+}/Mg^{2+}) with BSA (w/v 0.1%) (HBSS) and EGTA (1mM). The abdomen was gently massaged for 10-15seconds before the removal of fluid with a sterile plastic pastette. Cells were pelleted (400g, 10min at 20°C) and the blood cells were removed by hypotonic lysis with ice cold sterile distilled water (0.5ml) and made up to 25ml with buffer. Cells were then resuspended at $5x10^7$cells/ml in Percoll (1.070g/l) with BSA (1%). Lavaged cells were approximately 25% eosinophils and these were subsequently purified by centrifugation (1500g, 25 min at 20°C) on a discontinuous isotonic Percoll gradient with densities 1.070, 1.080, 1.085, 1.090 and 1.100g/l (10) made as detailed in table 2.1 below. One Percoll gradient was required for $1x10^8$ leukocytes.

Table 2.1 Protocol for Percoll gradients

Solution (g/l)	Volume/ gradient (ml)	2 Gradients		4 Gradients		6 Gradients	
		Light Percoll† (ml)	1x HBSS‡ (ml)	Light Percoll (ml)	1x HBSS (ml)	Light Percoll (ml)	1x HBSS (ml)
1.100	2.0	4.0	1.03	8.0	2.06	12.0	3.09
1.090	3.0	4.8	2.19	10.29	4.68	15.09	6.87
1.085	2.0	3.13	1.88	6.26	3.76	9.39	5.64
1.080	2.0	2.84	2.16	5.68	4.32	8.52	6.48
1.070	2.0	2.27	2.73	4.54	5.46	6.81	8.19

†Light Percoll = 9 parts Percoll + 1 part 10XHBSS with Ca^{2+}/Mg^{2+} pH 7.2-7.4 (store 4°C). ‡1XHBSS = HBSS with Ca^{2+}/Mg^{2+} pH 7.2-7.4.

Figure 2.1 Schematic representation of Percoll gradients



Cells were removed sequentially and the eosinophils were removed at the 1.090/1.100 g/l density interface and washed once in HBSS with EGTA (1mM) and then twice in HBSS. Approximately $2x10^7$ eosinophils/animal of >90% purity as assessed by Leishman's stain and >99% viability determined by trypan blue exclusion

were isolated.

2.2.1 Sensitization protocol for guinea pigs - bronchoalveolar eosinophils

Sensitiztion was performed as described by Watson *et al* (206). One day before primary sensitization, Dunkin-Hartley guniea pigs (400-600g, male and female) were injected with cyclophosphamide (1mg/kg, ip.) Animals were then sensitized by injection of ovalbumin (OA) (10μg/animal, ip.) with pertussis vaccine (0.25ml) and aluminium hydroxide (1mg/ml) as adjuvant, in a total volume of 1ml. Animals were boosted after 3 and 6 weeks with identical injections. Six to nine days later, conscious animals were restrained with snout in a plexiglass cone connected to a nebulizer. Animals were challenged by exposure to an aerosol of OA (10ml of 0.1% OA in physiological saline) or an aerosol of saline (10ml; sham-challenged) given over 1 hour. A control group of animals that were sensitized but not challenged by aerosol was also performed.

Twenty-four hours after the above treatments, animals were sacrificed by a lethal overdose of Euthatal (100mg/kg, ip.). Lung cells were collected by bronchoaleveolar lavage (BAL) with 4 x saline/BSA (0.025%) (20ml). Eosinophils were then purified as described in section 2.2

2.3 Purification of human eosinophils

Human eosinophils were purified by a protocol slightly modified from that of Hansel *et al* (207). 100ml of heparinised blood (4U/ml) was collected from a variety

of donors with informed consent. The whole blood was pelleted (1865g, 6min at 4°C) and the plasma was removed taking care not to disturb the leukocyte-rich layer at the interface. The red pellet was diluted to twice the original volume with RPMI 1640 with pen/strep and then layered onto Ficoll (35/15, v/v, blood/Ficoll) and centrifuged (450g, 30min at 20°C-no break to slow down). The peripheral blood mononuclear cells were removed and the granulocyte rich pellet was made up to the original volume (100ml) with RPMI 1640/EDTA (5mM) and layered onto dextran/saline 6% (1/1, v/v). This was allowed to sediment under gravity for least 1 hour. The granulocyte rich supernatent was then pelleted (350g, 10min at 4°C). The contaminating erythrocytes were removed by hypotonic lysis with ice-cold sterile distilled water (1ml) for approximately 30seconds then diluted to a final volume of 50ml with RPMI 1640/EDTA (5mM). The granulocytes were pelleted (200g, 10min at 4°C) and resuspended to $2x10^9$cells/ml in RPMI 1640/EDTA (5mM)/FCS (2%). The cells were incubated with CD16 microbeads $2x10^8$ granulocytes/100μl (30-45min on ice).

The MACS column was prepared as follows. First the column (type C) was flushed with ethanol and then RPMI 1640/EDTA (5mM) making sure no air bubbles entered the column. Next a 21Gx1½" needle with the tip removed with wire strippers but still in its plastic sheath was attached to the bottom of the column. Finally, the column was flushed through with RPMI 1640/EDTA (5mM)/FCS (2%) (30ml at 4°C).

Next the granulocyte preparation was made up to 1ml with RPMI 1640/EDTA (5mM)/FCS (2%). This was added to the top of the column and allowed to run into the column. The granulocytes were left in the column for 1-2min and then the column

was flushed through with RPMI 1640/EDTA (5mM)/FCS (2%) (30ml at 4°C). The CD16 negative cell elutant was collected as the eosinophil granulocyte population.

The MACS column was regenerated as follows (maximum of 6 times). The column was removed from the magnetic field and flushed through with RPMI 1640/EDTA (5mM)/FCS (2%) to remove the CD16 positive cells. Next the column was flushed through with RPMI 1640/EDTA (5mM) to remove any protein. The column was finally sterilised by flushing through ethanol. All the column openings were capped and then the column was stored upright at 4°C.

The eosinophils were pelleted (200g, 10min at 4°C) and resuspended in HBSS. Approximately $1x10^7$cells/100ml whole blood of >90% purity as assessed by differential cell count using Leishman's stain and >99% viability determined by trypan blue exclusion were isolated.

2.4 Culture of eosinophilic cell lines Eol-1 and Eol-3

The human eosinophil cell lines Eol-1 and Eol-3 were established from the peripheral blood of a patient with Philadelphia chromosome-negative leukaemia (185). Eol-1s and Eol-3s were a generous gift from Dr Mayumi, Kyoto University, Japan. The Eol's were maintained in RPMI 1640 supplemented with antibiotics (penicillin-streptomycin 100U/ml-100μg/ml) and FCS (10%). Cells were split twice a week to a cell concentration $5x10^5$cells/ml and cultured in vented culture flasks (500ml) at 37°C and 5% CO_2/95% air. Cell concentration increased to ~$1.5x10^6$cells/ml before splitting.

2.4.1 Maturation of eosinophilic cell lines Eol-1 and Eol-3

Cells were matured using various methods to try and establish cells as true to eosinophilic granulocytes as possible. Routinely the final differentiation step used was some from of butyrate. Either dibutyryl cAMP (0.2mM) or butyric acid (0.4-0.5mM) was added to freshly fed Eol cells. Cells were left to mature in flasks for 3,4,7 or 10 days without any additional feeding.

2.5 Measurement of hydrogen peroxide release

Hydrogen peroxide was determined by the oxidation of fluorescent scopoletin to a non fluorescent product at 37°C as previously described by Nathan (196). 96-well U-bottomed/flat-bottomed flexiplates were precoated with laminin (150μl/well, 3μg/ml) for an hour at 37°C. The plate was washed once with HBSS. To each well, cells (100μl at 2x10^6 cells/ml) with calcium/ magnesium (1.5mM) were added. Then buffer (50μl) and reaction mixture (10μl) containing scopoletin (3.6nmol), horseradish peroxidase (10μg), sodium azide (150nmol) and calcium/magnesium (20nmol) were also added. The plate was incubated at 37°C.

Stock solutions required for assay:

i) Scopoletin: 1.38mg/ml (7.2mM) in HBSS w/o BSA. 5mg dissolved in DMSO (50μl). Made up to volume with HBSS. Stored at RT and protected from light.

ii) Horseradish peroxidase: 20.0mg/ml. Stored at -20°C in 300μl aliquots.

iii) Sodium azide: 19.5mg/ml (300mM). Stored at RT.

iv) Calcium chloride: (200mM). Stored at -20°C in 1ml aliquots.

v) Magnesium chloride: (200mM). Stored at -20°C in 1ml aliquots.

Reaction Mixture:

250μl	Scopoletin
250μl	Horseradish peroxidase
250μl	Sodium azide
50μl	Calcium chloride
50μl	Magnesium chloride
4.15ml	HBSS

Test agonists (10μl) were added to start the reaction. The following agonists - PAF, C5a, LTB_4, fMLP, LPS, PMA; human cytokines - IL-5, GM-CSF, IFNγ, TNFα and human chemokines IL-8, NAP-2, MCP-1, MCP-3, MIP-1α, MIP-1β and RANTES were tested (10pM-100nM).

H_2O_2 release was detected as a decrease in fluorescence (excitation 355nm and emission 460nm) using a Titertek II platereader (ICN Biomedicals). A time zero reading was taken immediately before addition of the test agent, and then every 5min for 30min and subsequently every 15min for a further 1-2 hours. Controls were also performed in the presence of catalase (3000U/ml) and in the absence of horseradish peroxidase. In each assay, agents were tested in replicates of 3-4.

2.5.1 Calibration of H_2O_2 production

The amount of H_2O_2 was calibrated by adding known amounts of H_2O_2 to the reaction mixture. Two types of calibration curves were obtained.

type a) To blank wells containing buffer (150μl) and reaction mixture (10μl), a range of H_2O_2 standards (10μl) (0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6 nmols) were added. The standards were made up as follows:

i) 100μl H_2O_2 (30%) to 8.7ml of milli-Q distilled H_2O (100mM H_2O_2).

ii) 100μl of 100mM H_2O_2 to 9.9ml of milli-Q distilled H_2O (1mM H_2O_2).

iii) 50μl 1mM H_2O_2 to 950μl milli-Q distilled H_2O (50μM or 0.5nmol/10μl) *etc.*

Standards stored at 4°C and remade weekly.

type b) H_2O_2 standards (10μl) were also added to wells that contained eosinophils (100μl), buffer (50μl) and reaction mixture (10μl). This produced an identical curve but with a vertical shift, due to the auto fluorescence of eosinophils

For each assay, if cells allowed, a *type b* standard curve was done. If however cells were limited, as was frequently the case for human eosinophils, a *type a* standard curve was performed and the whole curve was shifted by a factor determined by the difference in fluorescence of cells with vehicle control compared to no cells with 0nmol H_2O_2.

To calculate the H_2O_2 produced by the samples, an exponential curve equation (2.1) was fitted to the standard H_2O_2 values.

$$y = y_0 + Ae^{-\left(\frac{x+x_0}{t}\right)} \quad (2.1)$$

$$e^{-\left(\frac{x+x_0}{t}\right)} = \frac{y-y_0}{A} \quad (2.2)$$

$$-\frac{x+x_0}{t} = \ln\left(\frac{y-y_0}{A}\right) \quad (2.3)$$

$$x = \left(-\ln\left(\frac{y-y_o}{A}\right)\right).t - x_o \quad (2.4)$$

Rearranging this equation and as $x_0=0$ equation 2.4 becomes:

$$x = \left(-\ln\left(\frac{y-y_o}{A}\right)\right).t \quad (2.5)$$

where x = nmol of H_2O_2 produced by the cells; y = remaining fluorescence after the cells have released H_2O_2; y_o, A and t are constants determined when fitting the standard curve. The spreadsheet Excel (version 5) was used to manipulate the data and calculate the H_2O_2 produced from the changes in fluorescence.

2.5.2 The effect of PKC inhibitors

In order to determine the effects of protein kinase C (PKC), a specific PKC inhibitor, Ro 31-8220/002 (142) (Dr Nixon, Roche) was used. After addition of the cells to the microtitre plate, either vehicle or Ro 31-8220/002 (0.3-3μM) (50μl) was incubated with the cells (20min at 37°C) before monitoring H_2O_2 production in response to added agonist.

2.5.3 Extracellular divalent cations and calcium influx experiments on H_2O_2 production

In order to determine the effects of extracellular divalent cations, H_2O_2 experiments were performed in the presence of calcium/magnesium (1mM), calcium only (1mM), magnesium only (1mM), the specific calcium chelator EGTA (1mM) and the nonspecific chelator EDTA (1mM). Also experiments were carried out in the presence of nickel/ calcium (1mM).

To investigate the role of calcium influx, cells were incubated in the presence and absence of the receptor operated calcium channel (ROCC) inhibitor, SK&F 96365 (10-100μM) (10min at 37°C).

2.5.4 The effect of tyrosine kinase inhibitors on H_2O_2 production

To determine the role of tyrosine kinase on H_2O_2 production, cells were incubated (2-4 hours at 37°C) in the presence or absence of the specific tyrosine inhibitors, genistein, herbimycin A, erbstatin and tyrphostin A-47 (1-100μM) before the addition of the agonist and monitoring of H_2O_2 production. Due to the fluorescent

nature of these compounds, a standard calibration curve in the presence or absence of these tyrosine kinase inhibitors was also performed.

2.5.5 The effect of wortmannin on H_2O_2 production

To determine the role of phosphoinositol 3-kinase (PI 3-kinase) in eosinophil activation, cells were incubated (10min at 37°C) in the presence or absence of the PI 3-kinase inhibitor, wortmannin (3-30nM) before monitoring H_2O_2 production (183).

2.5.6 NADPH oxidase in H_2O_2 experiments

To determine that the H_2O_2 produced from eosinophils was via the NADPH oxidase complex cells were incubated (30min at 37°C in the presence or absence of the NADPH oxidase inhibitors diphenyleneiodonium (DPI) (10μM) (94) and 4'-hydroxy-3'-methoxy-aceto-phenone (HMAP) (10μM) (208). Due to the fluorescent nature of these compounds, a standard calibration curve in the presence or absence of these inhibitors was also performed.

2.5.7 Cytochrome c assay

In addition another experiment to confirm H_2O_2 production was produced from the superoxide ion (O_2^-), experiments were performed to measure the superoxide release via the inhibition of cytochrome *c* reduction. 96-well flat bottomed flexiplates were precoated with laminin (100μl) (3μg/ml for 1 hour at 37°C). The plate was washed with HBSS. To each well cytochrome *c* (50μl) (100μM), test agents (50μl), superoxide dismutase (SOD) (100U/ml) or buffer (50μl) were added. The reaction

was started by adding eosinophils (50μl) (4x10^{6}cells/ml). The release of O_2^- was detected as an increase in absorbance (550nm) compared with agonist and SOD, using a microtitre platereader. In each assay agents were tested in quadruplicate.

2.6 Measurement of eosinophil adhesion

Non-adherent cells were removed after the H_2O_2 assay by washing the plate once with HBSS. Adhesion was measured as described by Gamble (198). Briefly, rose bengal (200μl) (2.5mg/ml in phosphate buffered saline (PBS)) was added. After 5min the plate was gently washed three times with PBS. After removal of the PBS the remaining adherent cells were then lysed with ethanol/PBS (100μl) (v/v 1/1) for 30min. The absorbance was measured in a microtitre platereader at an optical density of 540nm. Eosinophil adhesion was calibrated by staining 1x10^{8}eosinophils with excess rose bengal. These cells were washed seven times in PBS. The cells were resuspended at 2x10^{6}cells/ml. The eosinophils were then lysed with an equal volume of ethanol/PBS for 30min. The lysed suspension was then diluted to represent different cell concentrations, plated-out (100μl/well) and the absorbance was measured as describe above. This resulted in a linear relationship:

$$y = A + Bx \qquad (2.6)$$

Rearranging this formula, equation 2.6 becomes:

$$x = \frac{y - A}{B} \qquad (2.7)$$

where x = number of cells adhered to the plate; y = optical density of rose bengal dye

from lysed eosinophils; *A* and *B* are constants determined by linear regression when fitting the standard curve. The spreadsheet Excel (version 5) was used to manipulate the data to calculate the total number of eosinophils adhered to the microtitre plate.

2.6.1 Microtitre coating protein in the H_2O_2/adhesion assay

Initially when setting up the H_2O_2 and adhesion assays, an investigation was needed to ascertain the best microtitre plate and which protein to coat the plate if necessary. The following plates were tested: Limbro Titertek plates (ICN Biomedicals Ltd), Nunc ELISA plates (Gibco-Life Sciences) and Falcon flexiplates. First the proteins were quantified to establish the amount of protein present using Bradford's reagent (bovine serum albumin was used as a standard). The following proteins were assessed: bovine serum albumin (100ng/ml-20mg/ml), foetal calf serum (0.1-100%), fibrinogen (10μg/ml-10mg/ml), fibronectin (100ng/ml-100μg/ml), gelatin (10μg/ml-10mg/ml) and laminin (10ng/ml-10μg/ml). Each protein (200μl) was added to a Titertek plate. Next, Bradford's reagent (50μl) (Bio-rad) was added. After 5min the solutions were aspirated and the optical density was measured on a Titertek platereader at 620nm.

The following proteins were then subsequently used and assessed in the H_2O_2 and adhesion assays: foetal calf serum (1-10%), fibrinogen (1-10mg/ml) and laminin (1-3μg/ml).

2.7 Measurements of intracellular free calcium ($[Ca^{2+}]_i$) elevation

Eosinophil or Eol $[Ca^{2+}]_i$ was measured by monitoring the fluorescence of eosinophils loaded with the calcium indicators fura-2 or fluo-3 (103,104). Fura-2 was used for the majority of the experiments. Fluo-3 was used when the tyrosine kinase inhibitors interfered with the fluoresece of fura-2.

2.7.1 Fura-2 loaded cells

Purified cells (10^7 cells/ml) were loaded by incubation with fura-2 acetoxymethyl ester (2.5μM) in HBSS (30min at 37°C), washed twice and resuspended at 10^6 cells/ml in HBSS, 1mM Mg^{2+} and 100μM Ca^{2+}. Aliquots (2ml) were dispensed into a quartz cuvette and the external calcium adjusted to 1mM. The fluorescence signals of stirred aliquots were monitored at 37°C in a Deltascan fluorimeter (PTI Inc., NJ, USA) with a slit width of 5nm and dual excitation wavelengths of 340nm and 380nm and a single emission wavelength of 510nm before and up to 5min after the addition of test agents (20μl). The $[Ca^{2+}]_i$ was calculated using PTI software and the ratio method (103) after calibration of the signal in the presence of digitonin (12.5μM) and EDTA (10mM) to obtain values of R_{max} and R_{min} respectively as shown in equation 2.8 below (where fura-2 $Kd_{37°C}$ = 2.24x10^{-7}M, λ_1 = 340nm, λ_2 = 380nm and V=1).

$$[Ca^{2+}]_i = kd_{37°C} \cdot \frac{(R - V.R_{min})}{(V.R_{max} - R)} \cdot \frac{No\ bound\ (\lambda_2)}{Full\ bound\ (\lambda_2)} \qquad (2.8)$$

where $Kd_{37°C}$ = dissociation constant for fura-2 at 37°C; $R = \frac{Signal\ (\lambda_1)}{Signal\ (\lambda_2)}$ and V = viscosity coefficient.

A typical experiment is shown schematically in figure 2.2

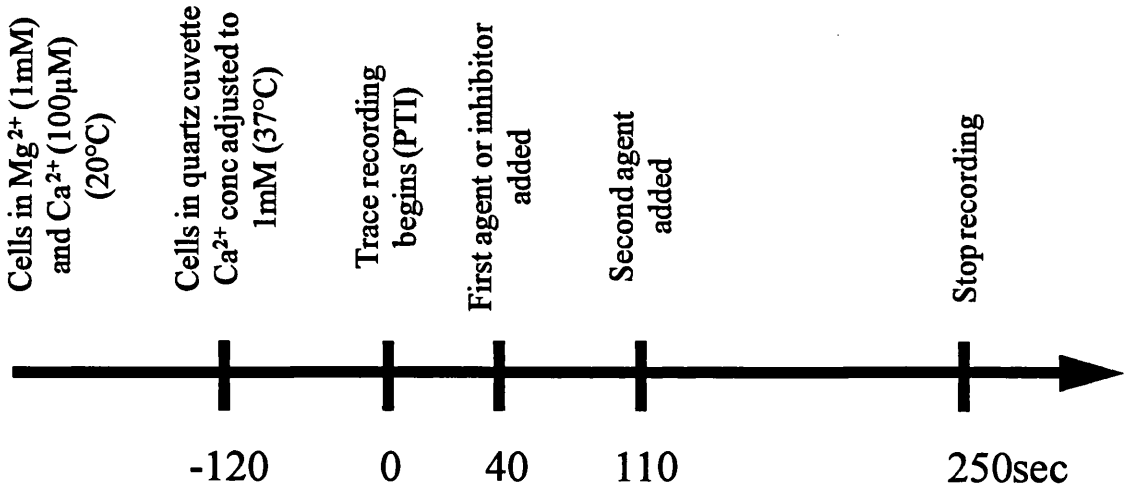


Figure 2.2 Schematic diagram of fluorimetery experiments

2.7.2 Fluo-3 loaded cells

Purified cells (10^7cells/ml) were loaded by incubation with fluo-3 acetoxymethyl ester (5μM) in HBSS (45min at 37°C), washed twice and resuspended at 10^6cells/ml in HBSS with Mg^{2+} (1mM) and Ca^{2+} (100μM). Aliquots (2ml) were dispensed into a quartz cuvette and the external calcium adjusted to 1mM. The fluorescence signals of stirred aliquots were monitored at 37°C with a single excitation wavelength 506nm and a single emission wavelength of 526nm before and up to 5 min after the addition of test agents (20μl). The calcium concentration was again calculated using PTI

software (fluo-3 $Kd_{37°C}$ = 4.0x10^{-7}M). Photobleaching of fluo-3 occurred rapidly, therefore loaded cells were protected from light at all times.

2.7.3 The effect of the PKC Inhibitors, Ro 31-8220/002

See section 2.5.2

2.7.4 Calcium influx experiments with nickel and manganese

Nickel and manganese could both be used to investigate the role of calcium influx. Aliquots (2ml) of fura-2 loaded cells (10^{6}cells/ml) were dispensed into a quartz cuvette. The fluorescence signals were monitored as described in section 2.7.1. $NiCl_2$ (1-5mM) was added after basal levels of $[Ca^{2+}]_i$ had been established. 60 seconds after addition of nickel, the test agent (20μl) was added and the signal was monitored.

Aliquots (2ml) of fura-2 loaded cells (10^{6}cells/ml) in HBSS, Mg^{2+} (1mM) and Ca^{2+} (100μM) were briefly pelleted (500g, 10secs) to remove any leaked fura-2 and resuspended in HBSS, Mg^{2+}(1mM) and Ca^{2+} (100μM) and dispensed into a quartz cuvette. The fluorescence signals of stirred aliquots were monitored at 37°C with dual excitation wavelengths 340nm and 360nm (fura-2 isobestic point) and a single emission wavelength of 510nm before and after addition of the test agent (20μl). 60 seconds after addition of the test agent, $MnCl_2$ (100-500μM) was added and the signal was monitored for a further 2 min. Finally the total calcium influx was measured by the addition of ionomycin (10μM).

2.7.5 The effect of SK&F 96365

Aliquots (2ml) of fura-2 loaded cells (10^6cells/ml) were dispensed into a quartz cuvette. The fluorescence signals were monitored as described in section 2.7.1. SK&F 96395 (10-100μM) or vehicle (H_2O) was added after basal levels of $[Ca^{2+}]_i$ had been established. Approximately 60 seconds post addition of SK&F 96365, test agents (20μl) were added and the signal monitored.

2.7.6 Tyrosine kinase experiments with the specific inhibitors genistein, herbimycin A, erbstatin and tyrphostin A-47

Due to the fluorescent nature of these compounds, first the effects of these tyrosine kinase inhibitors were investigated on free fura-2 acid and fluo-3 acid. Free fura-2 (100nM) or free fluo-3 (1μM) aliquots (2ml) were dispensed into a quartz cuvette and equilibrated to 37°C. Then after the addition of EGTA (2.5mM) to remove extracellular calcium, the tyrosine kinase inhibitors, genistein (100-300μM), erbstatin (1-100μM), herbimycin A (175nM-1.75μM) and tyrphostin A 47 (1-100μM) were added. The signal was monitored at the appropriate wavelengths for each fluorochrome as described in sections 2.7.1 and 2.7.2.

After establishing which tyrosine kinase inhibitors could be used with each fluorescent dye, fura-2 and fluo-3 loaded cells in HBSS with Ca^{2+}/Mg^{2+} (1mM) (2ml) were incubated to 37°C. The following inhibitors were then incubated with the cells; herbimycin A (875nM) in fura-2 and fluo-3 loaded cells and genistein (30-300μM) in fluo-3 loaded cells only. Guinea pig eosinophils were incubated with the inhibitors for 20 min. Eol-1s were incubated with the inhibitors overnight. Briefly, the tyrosine

kinase inhibitors were incubated with Eol-1s (10^6cells/ml) in media/culture flasks and loaded with fluo-3 just before investigating $[Ca^{2+}]_i$ elevation (due to the instability of fluo-3). The test agents (20μl) were then added and the signal was monitored at the appropriate wavelength for each fluorochrome as described in sections 2.7.1 and 2.7.2.

2.8 Measurement of tyrosine phosphorylation by western blotting

To establish whether tyrosine kinase was involved in the signalling pathways of eosinophils, a technique known as western blotting was employed.

2.8.1 Western blotting method

Cells were prepared as previously described and resuspended to 2.02x10^7cells/ml in HBSS without BSA. A final protein concentration of 10μg/20μl sample/lane for a mini gel or 100μg/100μl sample/lane for a large gel was required.

2.8.1.1 Preparation of western blotting samples

A 2x sample buffer (209), was prepared as described in table 2.2 below. Cells (297μl) were dispensed into an aggregometer tube and stirred with a magnetic flea (1min at 37°C). The calcium concentration was then adjusted to 1mM by adding 3μl of $CaCl_2$ (100mM) and the cells were incubated for another two minutes.

Test agonist or similar (30μl) was added at t=0s. After addition of the test agent typically 2s→5min, the reaction was stopped by adding 2x sample buffer (300μl at 100°C). Due to the large amount of nucleic material, the samples were sheared 5

times through a 1ml insulin syringe (27G). The samples were then boiled for 5 min, allowed to cool and stored at -20°C until required.

Table 2.2 2x sample buffer

Component	Volume	Final ratio/ concentration
10% SDS	3.0ml	(2%)
Glycerol	3.0ml	(20%)
1M Tris pH 6.8	2.4ml	(16%) 160mM
Distilled H_2O	5.1ml	(53%)
2-mercaptoethanol	1.35ml	(9%)
Bromophenol Blue	1 lump	-

Molecular weight markers (15,000→200,000) were made up by adding distilled H_2O (0.5ml) to the lyophilised markers. The markers were boiled for 5 min, allowed to cool, aliquoted (to avoid repeated freeze/thawing) and stored at -20°C until required.

2.8.1.2 Preparation and running of PAGE/SDS gels

The running gels were prepared as described in table 2.3 and table 2.4 below. The APS and TEMED were added just before the gel was poured.

Table 2.3 Protocol for 10%/15% running and stacking gels

Components	10% Gel		15% Gel		Stacking Gel	
	2 Large (ml)	1 Large/ 2mini (ml)	2 Large (ml)	1 Large/ 2mini (ml)	2 Large (ml)	1 Large/ 2mini (ml)
Protogel	20	10	30	15	1.7	0.85
1M Tris pH 8.8	22.5	11.25	22.5	11.25	-	-
1M Tris pH 6.8	-	-	-	-	1.25	0.625
H_2O	16.3	8.15	6.3	3.15	6.84	3.42
10% SDS	0.6	0.3	0.6	0.3	0.1	0.05
10% APS	0.6	0.3	0.6	0.3	0.1	0.05
TEMED	0.024	0.012	0.024	0.012	0.01	0.005
Total	60	30	60	30	10	5

60ml was sufficient for 2 large gels
30ml was sufficient for 1 large gel or 2 mini gels

For gradient gels the following protocol was used:

Table 2.4 Protocol for gradient running and stacking gels

Components	7% (ml)	17% (ml)	Stacking gel (ml)
Protogel	3.5	8.5	1.6
1M Tris pH 8.8	5.6	5.6	-
1x Upper Buffer	-	-	3.1
Sucrose	-	1.5g	-
H_2O	5.8	-	7.6
10%SDS	0.075	0.075	-
10% APS	0.15	0.15	0.12
TEMED	0.15	0.15	0.12
Total	30		12.5

4x Upper buffer (stock): 6.06g Trizma Base and10% (4ml) SDS; Dissolved in H_2O (85ml), pH 6.8 with 1M HCl, then made up to 100ml. Store at 4°C.

Made up to 1x Upper buffer with distilled water before pouring gel.

The gel apparatus - large (Höefer Scientific Instruments, Newcastle-Under-Lyme, UK) and mini (Bio-Rad laboratories, Hemel Hempstead, UK) were cleaned with methanol before use. First the running gel was poured to approximately 0.5cm below the point where the bottom the combs would reach. 50% methanol was layered on the top of the gel until set. When set, the methanol was washed off, the plastic combs were placed in position and then the stacking gel was poured on top of the running gel, making sure no air bubbles were trapped. When the stacking gel had set, the combs were carefully removed and the top of the gel was washed with distilled water. Running buffer was then applied to the top of the gel.

Samples (100μl - large or 20μl - mini) were added to individual lanes. Molecular weight markers were applied to at least one lane in each gel (7.5μl - large or 3μl - mini). The gel apparatus was then assembled for running and power was passed through the gel using a suitable power pack at the appropriate constant voltage, as shown in table 2.5 below.

Table 2.5 Voltage and time guide for the running of PAGE/SDS gels

Gel	Stacking gel		Running gel	
	Large	Mini	Large	mini
Voltage	50V	50V	50V	150V
Time	15min	15min	Overnight	1-1½ hours

When the bromophenol blue dye reached the bottom of the gel, the power supply was disconnected. If the gel was to be stained to indicate the position of the proteins, the gel was placed in Coomaissie blue stain for at least 2 hours.

2.8.1.3 Transfer of proteins from gels to nitrocellulose membranes

Rubber gloves were worn to avoid protein contamination from hands. Filter card and Immobilon-P transfer nitrocellulose membranes were cut to match the size of the gel. The gels were transferred to the nitrocellulose membrane using a Trans-Blot with transfer cells (Bio-Rad Laboratories, Hemel Hempstead, UK) as follows:

1) A transfer cell was opened, then a transfer pad soaked in transfer buffer was placed on the black side of the transfer cell.

2) Next a soaked filter card was placed on the transfer pad. At each stage, all the air bubbles were removed by rolling them out with a glass pasteur pipette.

3) The gel was then carefully placed on the filter card.

4) Next a piece of nitrocellulose paper, activated by immersing in methanol (3 sec), distilled H_2O (1 min) and transfer buffer (1 min), was placed on the gel.

5) On top of the membrane, another filter card soaked in transfer buffer was

placed.

6) On top of the filter card, another transfer pad soaked in transfer buffer was placed.

7) Finally the cell was closed (nb. clear side of cell was on top of the final pad).

The transfer cell was then placed in the Trans-Blot with the black side of the cell facing the cathode side of the tank and the clear side facing the anode. It was important to get this the correct way round, otherwise the proteins would move towards the bottom filter card not the membrane. The above was repeated for each gel. The Trans-Blot was then assembled, filled with transfer buffer to the top and placed on a magnetic stirrer. The blots were then transferred at constant current using a suitable power pack as shown in table 2.6 below.

Table 2.6 Current, time and temperature guide for transferring gels to nitrocellulose membranes

Gel	Large	Mini
Voltage	50V	maximum
Current (constant)	0.2A	0.333A
Time	Overnight (min 20 hours)	1¼-1½ hours
Temperature	4°C	Ice pack in transfer tank

After transferring, the transfer cell was dismantled and the membrane was put into a suitable container, with the protein side facing upwards ready for probing with appropriate antibodies (the membrane can be stored in PBS/0.1% NaN_3 at 4°C overnight). The gel was then stained in Coomassie blue, to mark proteins left behind on the gel.

2.8.1.4 Blocking and probing of membranes

All steps were carried out at room temperature. First the nonspecific proteins were blocked using a suitable agent. Each membrane was covered with non-fat milk protein in PBS (5%), (200ml - large or 50ml - mini) and left on a shaker for 1½ - 2 hours. Alternative blocking agents included gelatin (3%) and serum (5%). Blocked membranes were washed 3 times with PBS/Tween 20 (0.05%) for 10 min on a shaker.

The membranes were then probed with the anti-phosphotyrosine antibodies PY20 (1/500 - 1/5000 dilution) or 4G10 (1/500 - 1/5000 dilution) in non-fat milk protein (0.5%)/NaN_3 (0.1%)/PBS for 2 - 3 hours on a shaker. After probing, the antibody was removed to be reused for a maximum of 3 times, filtering each time before reuse.

The membranes were then washed 3 times with PBS/Tween 20 (0.05%) for 10 min on a shaker. The membranes were then incubated with a goat anti-mouse IgG horseradish peroxidase conjugate antibody (1/500 - 1/2000 dilution) in non-fat milk protein (0.5%)/PBS for 1¼ - 1½ hours. The membranes were then washed 3 times with NaCl (500mM)/PBS/Tween 20 (0.05%) for 15 min and then twice with PBS/Tween 20 (0.05%) for 15 min.

Control first antibodies used:

- mouse whole IgG (1/500 - 1/5000) - to indicate if nonspecific binding of PY20/4G10 was binding to eosinophils.
- o-Phospho-DL-serine (1mM) with PY20/4G10, o-Phospho-DL-threonine (1mM) with PY20/4G10, o-Phospho-DL-tyrosine (1mM) with PY20/4G10 - to demonstrate the antibody was only marking tyrosine phosphorylated proteins.

Other control experiments included:

- No first antibody (ie. non-fat milk protein (0.5%)/NaN_3 (0.1%)/PBS)
- No second antibody (ie. non-fat milk protein (0.5%)/PBS)

2.8.1.5 Detection of tyrosine phosphorylation using enhanced chemiluminescence (ECL)

The PBS/Tween 20 (0.05%) was poured off from the membrane. An equal volume of reagent A was mixed with reagent B to give sufficient reagent to cover the membrane (final volume 0.1ml/cm^2). The ECL reagent was incubated with the membrane for precisely 1 min at room temperature. The excess detection reagent was drained off and the blot was wrapped in Saranwrap, smoothing out any air pockets and sealing the edges with masking tape. This was repeated for each membrane. The blots were then immediately exposed to Hyperfilm-ECL for 15 sec and processed using a RGII Film processor (Fuji Photofilm Co. Ltd). A second exposure was then performed (1 min - 1 hour). The length of the second exposure was assessed according to the amount of protein that appeared on the first exposure.

2.8.1.6 Ponceau S staining of the nitrocellulose membranes

To stain the proteins on the membrane, temporarily or permanently, the stain ponceau S was used. Enough ponceau S (stock diluted in distilled H_2O) to cover the blot was added for approximately 2 min. The excess ponceau S was drained off, then enough PBS was added to liberally cover the blot. The blot was shaken for approximately 3 min, then replaced with fresh PBS. The protein bands were then

clearly visible. To stain permanently, after draining the excess ponceau S, acetic acid (1%) was added for 2 min. The blot was then allowed to air dry.

2.8.1.7 Destaining and drying gels

Excess Coomaissie stain was poured off the gels and back into stock supply. The gels were then covered with destain (methanol/acetic acid/distilled H_2O, v/v/v, 40/7/53). The gels were left on a shaker for about 2 hours replacing the destain as required. Finally the destain was replaced with distilled H_2O.

A piece of filter paper cut to the size of the gel, soaked in H_2O was placed in a gel dryer (Bio-Rad laboratories, Hemel Hempstead, UK). The gel was then carefully placed on the filter paper and covered with a piece of cellophane membrane soaked in H_2O. This was repeated for each gel to be dried. The plastic seal of the dryer was put over the top of the gels and the lid closed. The gel was dried for 1 hour 10 min at 80°C under vacuum. The vacuum pump was left running for ½ hour after the gels had finished drying while the equipment cooled down. The dried gels were then removed before switching off the vacuum pump.

2.8.2 Experiments with the specific tyrosine kinase inhibitors genistein, herbimycin A, erbstatin and tyrphostin A-47

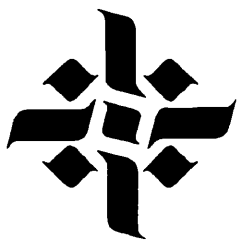
To try and inhibit cellular function via tyrosine phosphorylated proteins, the specific tyrosine kinase inhibitors, genistein, herbimycin A, erbstatin and tyrphostin A-47 were used. Eosinophils were prepared and resuspended at $2x10^7$cells/ml with Ca^{2+}/Mg^{2+} (1mM) and incubated with the tyrosine kinase inhibitors for 1 hour at 37°C

(not stirred). For Eol-1s overnight incubations were performed. Briefly, the tyrosine kinase inhibitors genistein (30μM) and herbimycin A (875nM) were incubated with the cells (10^6cells/ml) in media and culture flasks. The cells were washed twice in HBSS without BSA, resuspended at $2x10^7$cells/ml with Ca^{2+}/Mg^{2+} (1mM).

The cells were then incubated with test agents as described in section 2.8.1.1, stopping the reaction with 2x sample buffer. The samples were then used for immunoblotting.

2.9 Statistical analysis

Minitab for windows (version10) was used for all statistical analaysis. Unless otherwise stated, a 2 way ANOVA was performed on the groups of data and the significance was calculated using the Dunnett's method. In the result sections, all the figures have the same legend; significant increases above basal levels: *, $p<0.05$; **, $p<0.01$ and significant decreases/inhibitions compared with vehicle controls: #, $p<0.05$; ##, $p<0.01$.



3 Eosinophil Hydrogen Peroxide Release

3.1 Purity of eosinophils

3.1.1 Guinea pig eosinophils

As stated in the protocol for isolation of guinea pig eosinophils, $2x10^7$ cells/animal were obtained of >90% purity and >99% viability. In the results section, no data was used from any experiment if the purity was <90%. The main contaminating cells were macrophages. Very few neutrophils or lymphocytes (<1%) were present. A typical purified eosinophil preparation is shown in plate 3.1.

3.1.2 Human eosinophils

As stated in the protocol for isolation of human eosinophils, typically $1x10^7$ cells/100ml whole blood were obtained of >90% purity and >99% viability. In the results section, no data was used from any experiment if the purity was <85%. The lower number of human eosinophils obtained was the reason behind accepting a less pure preparation in the data section. The contaminating cells were more varied in the human eosinophil preparations. Monocytes and lymphocytes were the main contaminants with neutrophils and basophils (<3%) also being present.

3.2 Setting up the hydrogen peroxide assay

As outlined in the method section, different types of microtitre plates and protein coatings were investigated for their suitability with this assay. In any plate used, if there was no protein coating, the cells adhered to the plate and were activated to produce H_2O_2 from eosinophils without stimulation by an added agonist. Therefore the following proteins were tested: BSA; FCS; fibrinogen; fibronectin;gelatin and

laminin. BSA, fibronectin and gelatin were discounted due to the large quantites needed for sufficient protein to coat the plate.

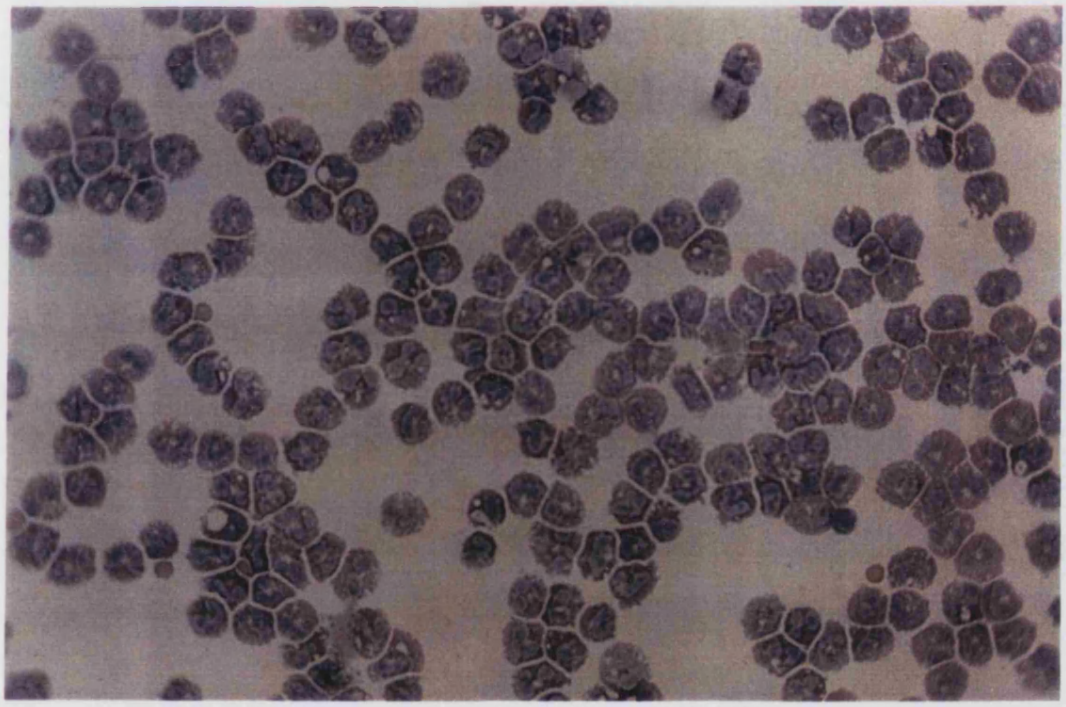


Plate 3.1 Photograph of purified eosinophils from a guinea pig

FCS did not adhere to the wells sufficiently to prevent activation of the eosinophils by the microtitre plate. Both fibrinogen (10mg/ml) and laminin (3μg/ml) prevented spontaneous activation of the eosinophils by the microtitre plate and allowed stimulation of the purified cells by added agonist (figure 3.1).

Regardless of the protein coating used, ELISA plates caused the cells to adhere and activate without stimulation. Figure 3.1 shows that laminin and fibrinogen gave measurable responses on either flexiplates or titertek plates. However the laminin coating caused a higher basal response on the titertek plate and fibrinogen lowered the response effected by C5a. Laminin-coated microtitre flexiplates was chosen as the

preferred protein coating and plate for subsequent experiments due the larger window of detection of H_2O_2 release when stimulated by C5a.

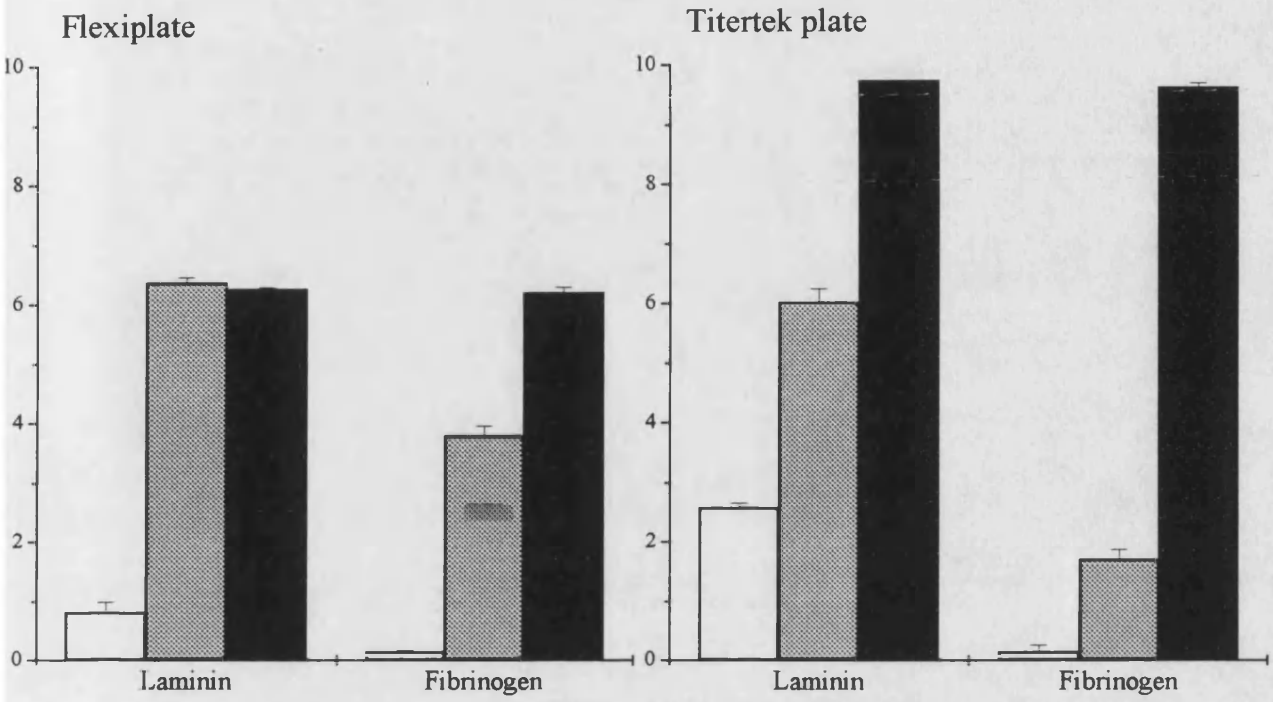


Figure 3.1 Hydrogen peroxide released from guinea pig eosinophils in either flexiplates or titertek plates. Eosinophils were either unstimulated (□) or stimulated with C5a (100nM)(▒) or PMA (100nM)(■) in laminin-coated (3μg/ml) or fibrinogen-coated (10mg/ml) microtitre plates at t=30min. Bars indicate mean±sem nmol H_2O_2 produced/2x10^5cells for quadruplicate wells. Representative of 2 experiments.

3.3 Guniea pig eosinophils

3.3.1 Effect of the added agonists C5a, LTB_4 and PAF

Figure 3.2 shows a representative set of standards calibrating the increasing amount of hydrogen peroxide on the reduction of the fluorescent compound scopoletin. An exponential curve was fitted to the standards using equation 2.1 to give the following typical parameters in table 3.1. A new H_2O_2 calibration was performed for each assay.

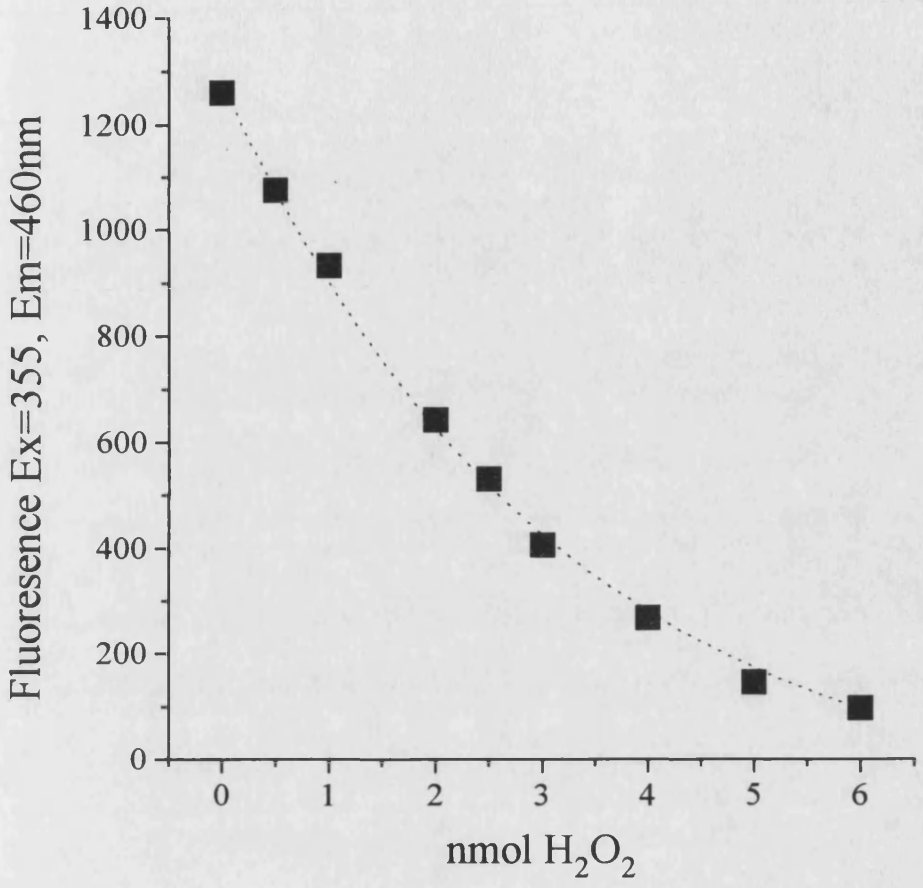


Figure 3.2 Representative standard curve of decreasing scopoletin fluoresence with increasing amounts of H_2O_2. Trace indicates fluoresence measured at an excitation wavelength 355nm and emmission wavelength 460nm.

Table 3.1 Parameters of exponentional fit for figure 3.2

Parameter	Value
x_o	0
y_o	-109.18
A	1388.50
t	3.14
Chisqr	2.92

Both C5a and LTB_4 were potent stimulators of hydrogen peroxide production

from guinea pig eosinophils causing a dose related release of H_2O_2, which was significant at 10nM and 100nM (figures 3.3 and 3.4) for both agonists compared with unstimulated cells. Although causing some H_2O_2 release above basal levels, PAF (1 - 100nM) was only a weak activator of H_2O_2 production. As it can be seen from figure 3.3, nearly all the H_2O_2 was released within the first 30 min, with initial rates being comparable for the different agonists. There also appeared to be no lag between addition of the agonist and the commencement of H_2O_2 production. The results in figure 3.4 demonstrate the pronouced effects of C5a and LTB_4 at 10nM and 100nM compared with the response to PAF. IL-8, NAP-2, fMLP and LPS were inactive in this assay (table 3.2).

Table 3.2 Effects of IL-8, NAP-2, fMLP and LPS on H_2O_2 production from guinea pig eosinophils at t=30min. Data indicates mean±sem for quadriplicate wells. Representative of 2-4 experiments.

Agonist	H_2O_2 produced (nmol/2x10^5cells), t=30min
Basal	0.58±0.21
IL-8 (10nM)	0.76±0.26
NAP-2 (100nM)	0.55±0.02
fMLP (100nM)	0.51±0.04
LPS (10μg/ml)	0.58±0.04
C5a (100nM)	5.59±0.03

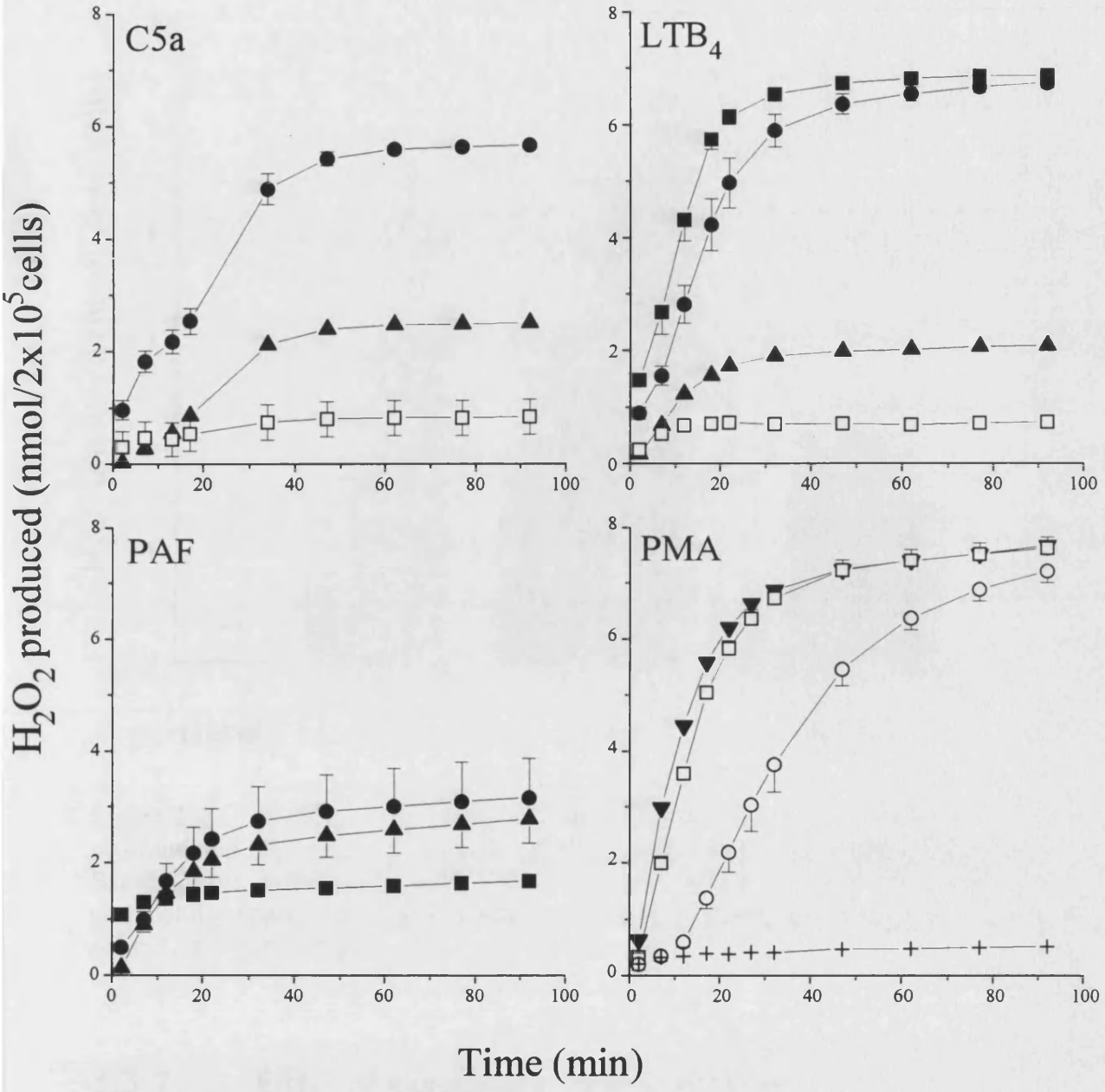


Figure 3.3 Time courses of H_2O_2 production from guinea pig eosinophils stimulated with different concentrations of C5a, LTB_4, PAF and PMA. Traces indicate mean±sem nmol H_2O_2 production from triplicate wells. Doses: 100pM (+); 300pM (○); 1nM (□); 3nM (▼); 10nM (▲); 100nM (●) and 1μM (■). Representative of 4-9 experiments.

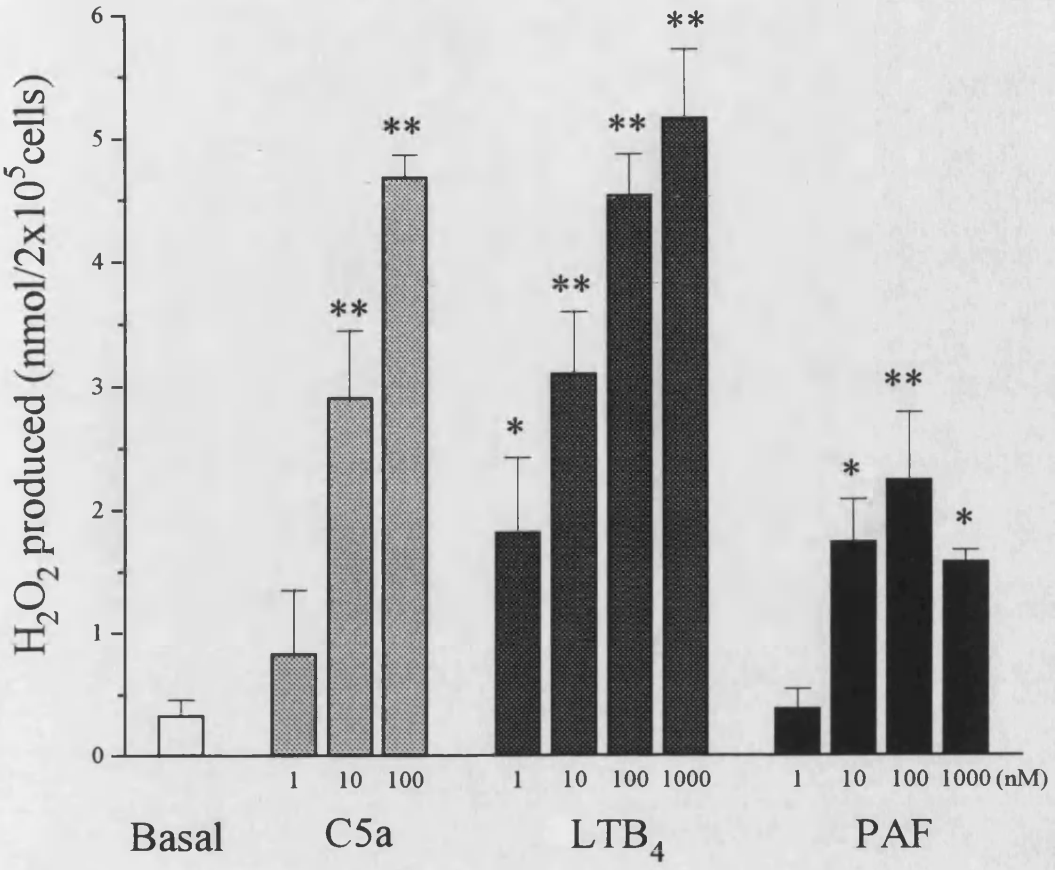


Figure 3.4 Hydrogen peroxide released from guinea pig eosinophils either unstimulated (□) or stimulated with C5a (▒), LTB_4 (▩) and PAF (■) at t=30min. Bars indicate mean±sem nmol H_2O_2 produced/ $2x10^5$cells for n=4-9 different eosinophil preparations. Significantly increased H_2O_2 compared with vehicle treated cells: *, $p<0.05$; ** $p<0.01$.

3.3.2 Effect of extracellular ionic conditions

The ability of eosinophils to produce hydrogen peroxide was greatly dependent on the extracellular cationic conditions. The calcium specfic chelator, EGTA, inhibited the H_2O_2 release stimulated with C5a, LTB_4 and PAF (100nM) by 53%, 52% and 63% respectively (figure 3.5).

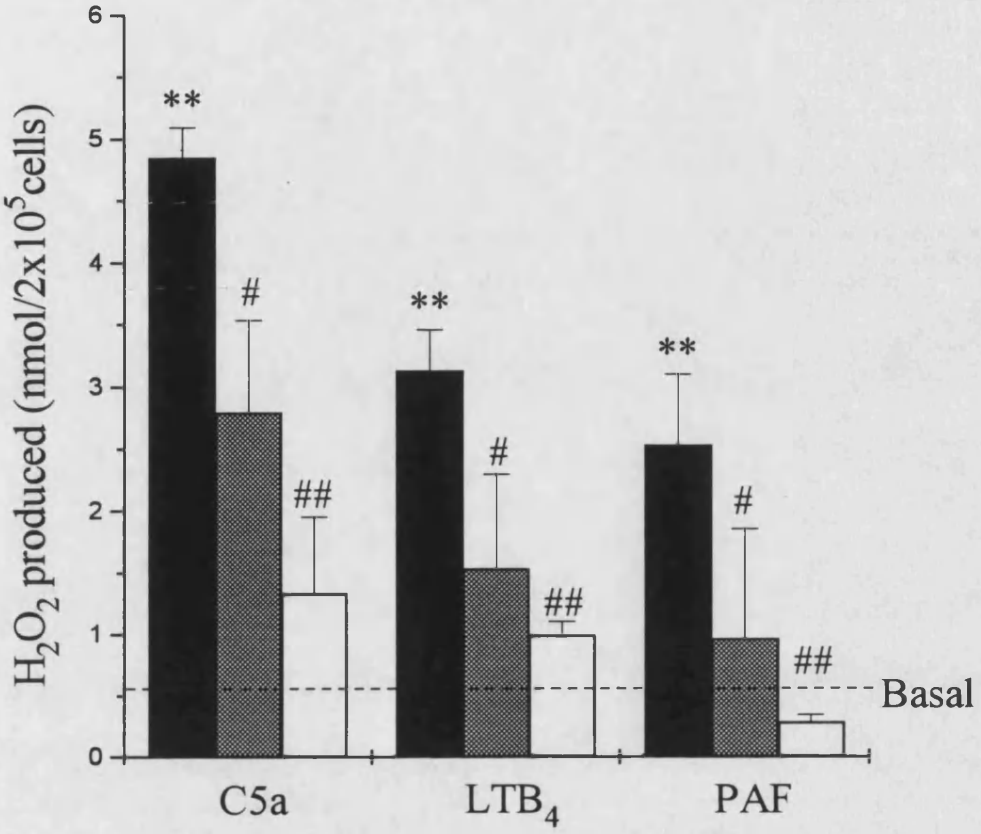


Figure 3.5 Hydrogen peroxide released from guinea pig eosinophils with C5a, LTB_4 or PAF (100nM) with varying extracellular cationic conditions at t=30min: (■) 1mM Ca^{2+}/Mg^{2+}; (▩) 1mM EGTA; (□) 1mM EDTA. Bars indicate mean±sem nmol H_2O_2 produced/2x10^{5}cells for n=4-9 different preparations. Significantly increased H_2O_2 produced compared with vehicle treated cells: *, $p<0.05$; **, $p<0.01$. Significantly reduced H_2O_2 produced compared with 1mM Ca^{2+}/Mg^{2+} containing cells: #, $p<0.05$; ##, $p<0.01$.

When the nonspecific chelator, EDTA, was tested it was found that H_2O_2 production by each agonist was inhibited by more than 80% (figure 3.5), indicating that both extracellular calcium and magnesium were required for H_2O_2 release. Experiments in stirred cells showed that 1mM EDTA prevented H_2O_2 production from guinea pig eosinophils by 30nM C5a (figure 3.5A). EDTA either completely inhibited H_2O_2 release when added at the start of the experiment or stopped the reaction in

progress when added during the course of the experiment (figure 3.8A). Figure 3.6 shows the decrease in fluorescence of the scopoletin-based reaction mixture in a cuvette detected by the PTI fluorimeter upon the addition of known amounts of H_2O_2. This was done to give a guide of the amount of H_2O_2 released from guinea pig eosinophils upon stimulation in the stirred-cell system.

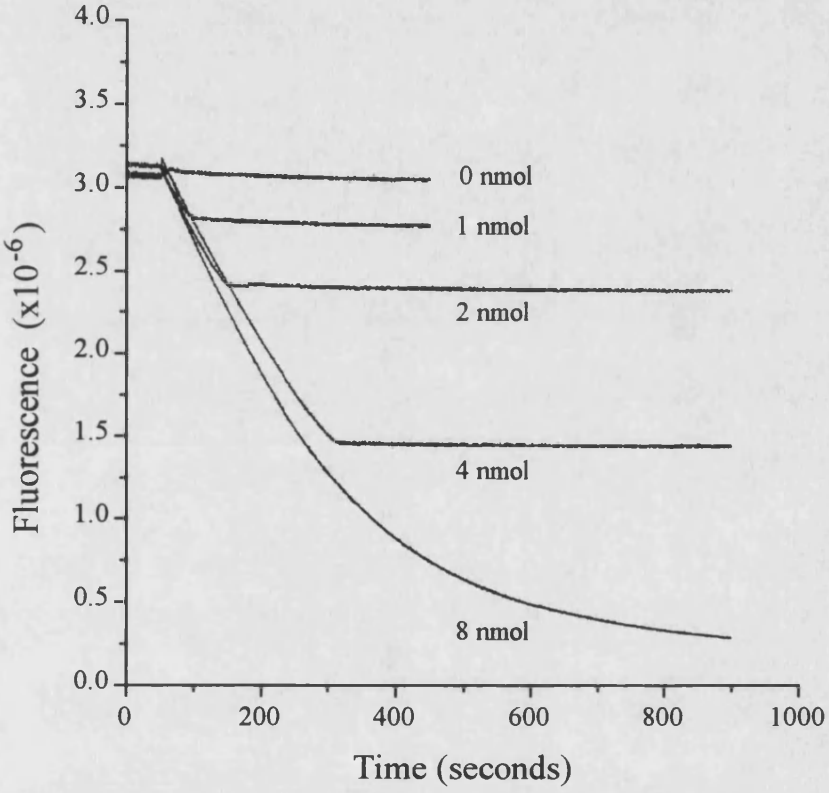


Figure 3.6 Effect of hydrogen peroxide on scopoletin fluorescence detected by PTI fluorimeter. Ex=355nm, Em=460nm.

3.3.3 Effect of Nickel on H_2O_2 release

Having established extracellular calcium and magnesium were important for the release of H_2O_2 from guinea pig eosinophils, it was next investigated to see if the calcium influx rather than intracellular calcium release was effecting H_2O_2 production. Figure 3.7 shows that when calcium/magnesium (1mM) and nickel (1mM) were present together, no effect on H_2O_2 production was observed when guinea pig eosinophils were stimulated with C5a, LTB_4 and PAF (100nM) (taking into account the increase in fluorescence caused by nickel chloride).

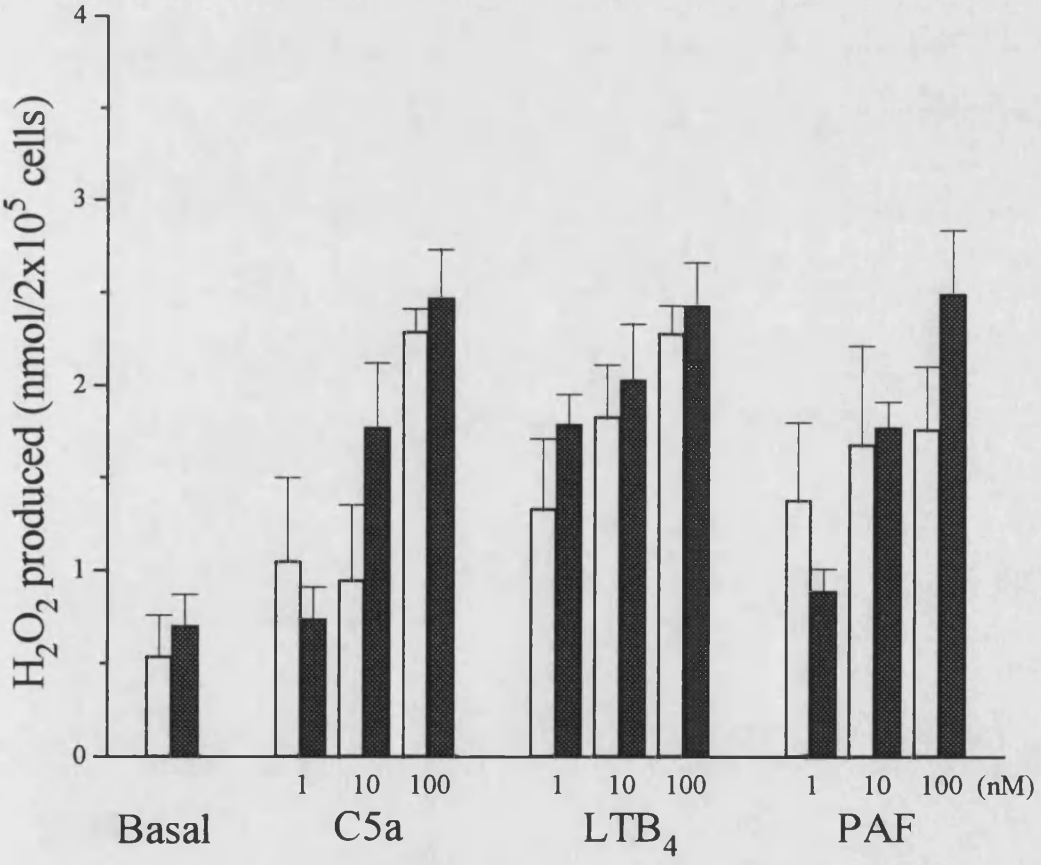


Figure 3.7 Hydrogen peroxide released from guinea pig eosinophils either unstimulated or stimulated with C5a, LTB_4 and PAF (1-100nM) t=30min. Cells stimulated under normal conditions (□) or in the presence of 1mM $NiCl_2$ (▩). Bars indicate mean±sem nmol H_2O_2 produced/2x10^{5}cells for triplicate wells. Representative of 2 experiments.

However, contrary evidence was obtained when the H_2O_2 experiments was carried out on a stirred suspension of cells in the fluorimeter. These experiments were performed to mimic the conditions used for $[Ca^{2+}]_i$ experiments (see chapter 5). Figure 3.8B shows that even once the cells appear to be producing H_2O_2, the addition of extracellular nickel (1mM), stops this process, suggesting influx does have a role in H_2O_2 production.

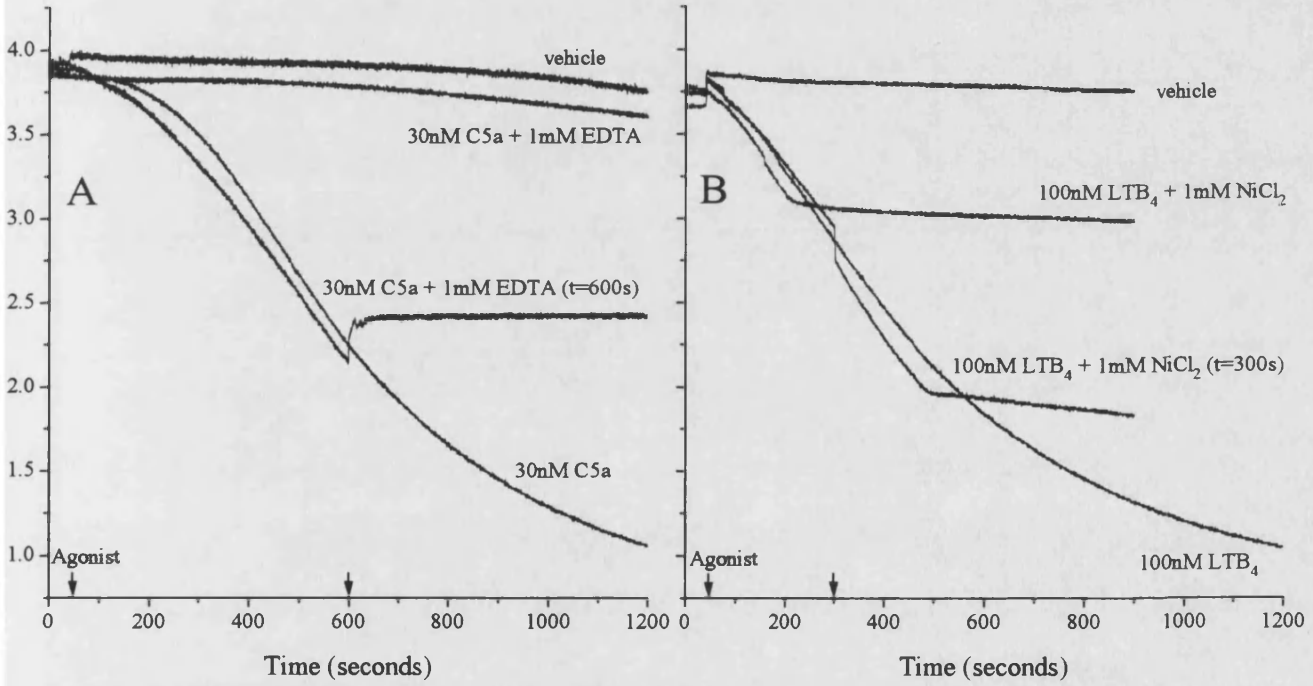


Figure 3.8 Hydrogen peroxide released from guinea pig eosinophils in stirred suspensions at 37°C. (A) Effect of EDTA (1mM) on cells stimulated with C5a (30nM). (B) Effect of $NiCl_2$ (1mM) on cells stimulated with LTB_4 (100nM). Traces are representative of 2 different eosinophil preparations.

3.3.4 Effect of SK&F 96365 on H_2O_2 release

To investigate further this role of calcium influx, the proposed specific receptor operated calcium channel (ROCC) blocker, SK&F 96365 (30-100μM) was used (117).

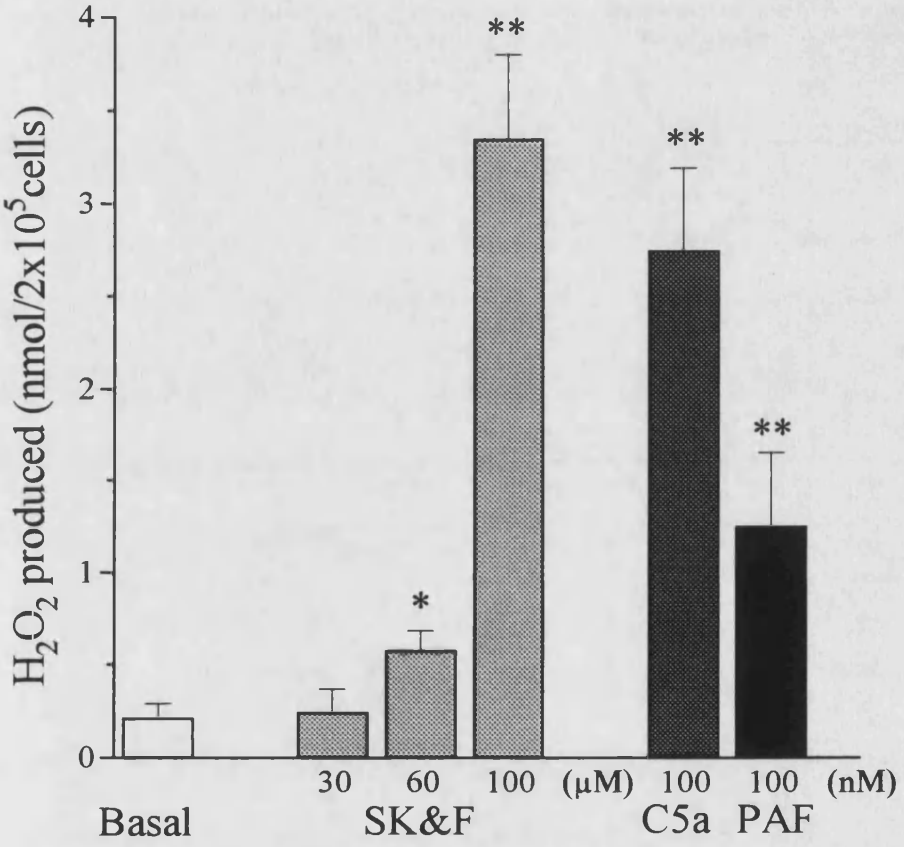


Figure 3.9 Hydrogen peroxide released from guinea pig eosinophils either unstimulated (□) or stimulated with SK&F 96365 (30-100μM)(▒), C5a (100nM)(▩) and PAF (100nM)(■) at t=30min. Bars indicate mean±sem nmol H_2O_2 produced/2x10^5 cells for n=5-6 different eosinophil preparations. Significantly increased H_2O_2 released compared with vehicle treated cells: * $p<0.05$; **, $p<0.01$.

Surprisingly, SK&F 96365 (60μM and 100μM) caused a significant release of H_2O_2 from guinea pig eosinophils (figure 3.9). The enantiomers of SK&F 96365 were also tested and figure 3.10 shows that each component was able to cause H_2O_2 release (significant at 100μM). However the combined values of H_2O_2 released by the enantiomers did not equate the values of H_2O_2 released by the racemic compound.

In conjunction with the added agonists C5a (100nM) and PAF (100nM), SK&F 96365 (30 - 100μM) significantly enhanced the release of H_2O_2 production from guinea pig eosinophils by a magnitude similar to the compound on its own (figure 3.11).

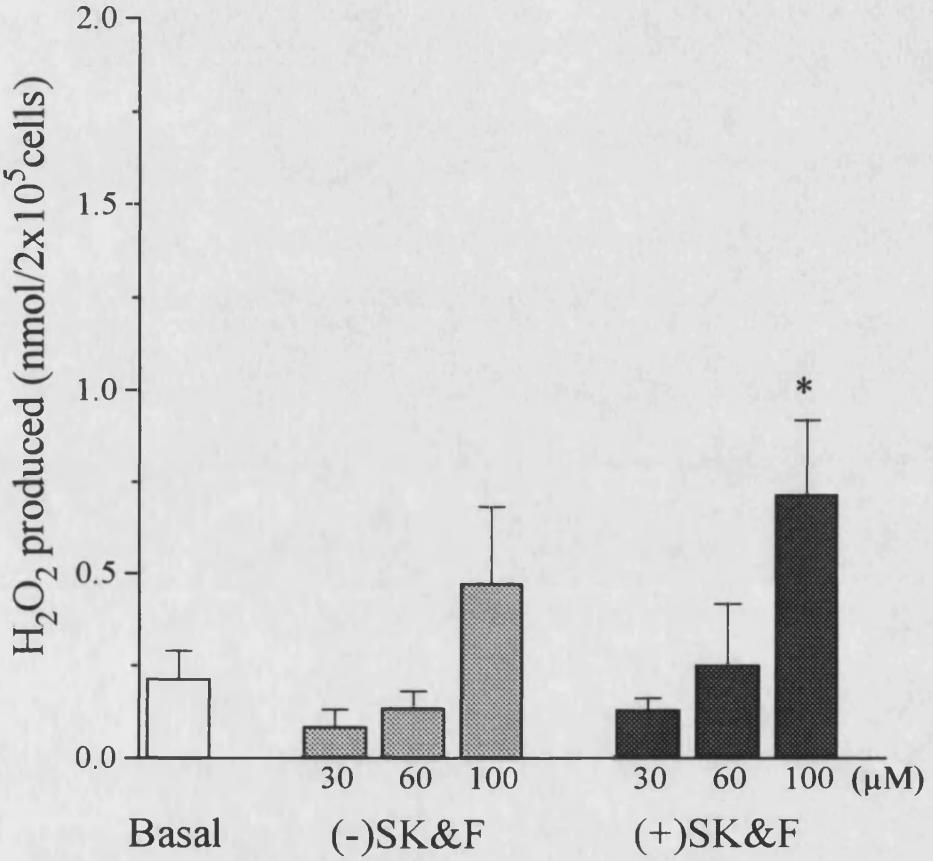


Figure 3.10 Hydrogen peroxide released from guinea pig eosinophils either unstimulated (□) or stimulated with the enantiomers of SK&F 96365: - (30-100μM)(▒); + (30-100μM)(▩) at t=30min. Bars indicate mean±sem nmol H_2O_2 produced/2x10^5 cells for n=4 different eosinophil preparations. Significantly increased H_2O_2 compared with vehicle treated cells: *, $p<0.01$.

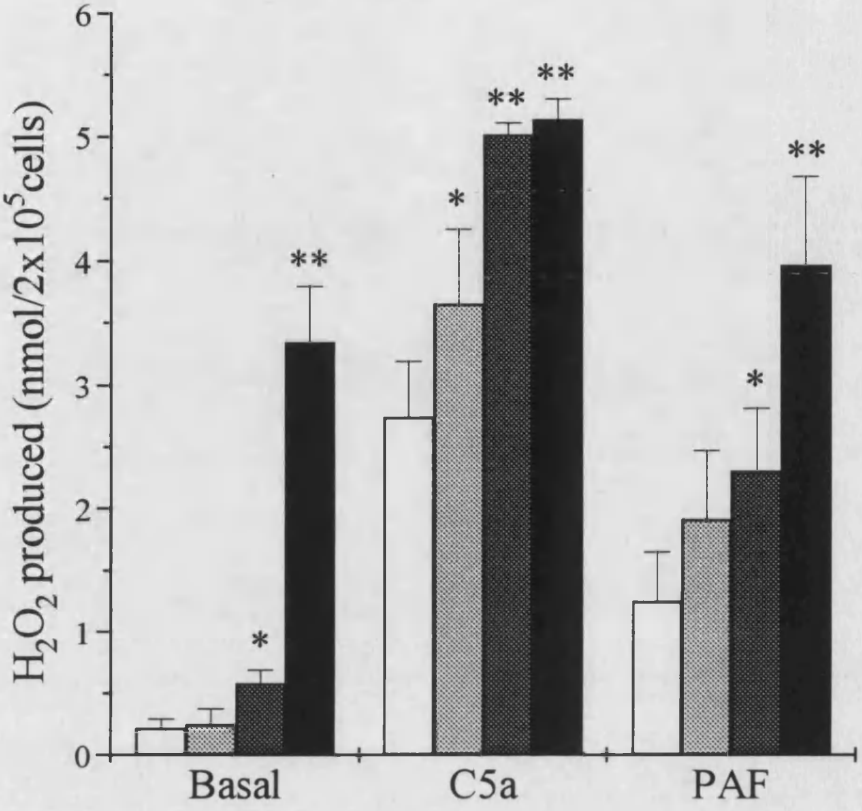


Figure 3.11 Hydrogen peroxide released from guinea pig eosinophils either unstimulated or stimulated with C5a (100nM) and PAF (100nM) in conjunction with SK&F 96365: vehicle (□); 30μM (▒); 60μM (▩) or 100μM (■) at t=30min. Bars indicate mean$\pm$sem nmol H_2O_2 produced/2x10^5 cells for n=4-5 different eosinophil preparations. Significantly increased H_2O_2 compared with vehicle treated cells: *, $p<0.05$; **, $p<0.01$.

3.3.5 Effect of the specific PKC inhibitor, Ro 31-8220/002

To investigate whether the agonists were stimulating H_2O_2 via a PKC mechanism, a selective PKC inhibitor, Ro 31-8220/002 was used in conjuction with suboptimal doses of C5a(30nM), LTB_4 (30nM) and PMA (1nM). PMA (1nM) was an extremely rapid activator of H_2O_2 production fom guinea pig eosinophils compared with C5a (100nM), and at a lower dose (300pM) the response, was less rapid, but still

produced the same amount of H_2O_2 (figure 3.3). PMA activates PKC directly and was therefore used as a positive control. Figure 3.12 demonstrates that both C5a, LTB_4 (30nM) and PMA (300pM) were dose-dependently inhibited by Ro 31-8220/002. Furthermore, not only reducing the final amount of H_2O_2 produced, Ro 31-8220/002 also affected the initial rate of the reaction in cells stimulated by PMA.

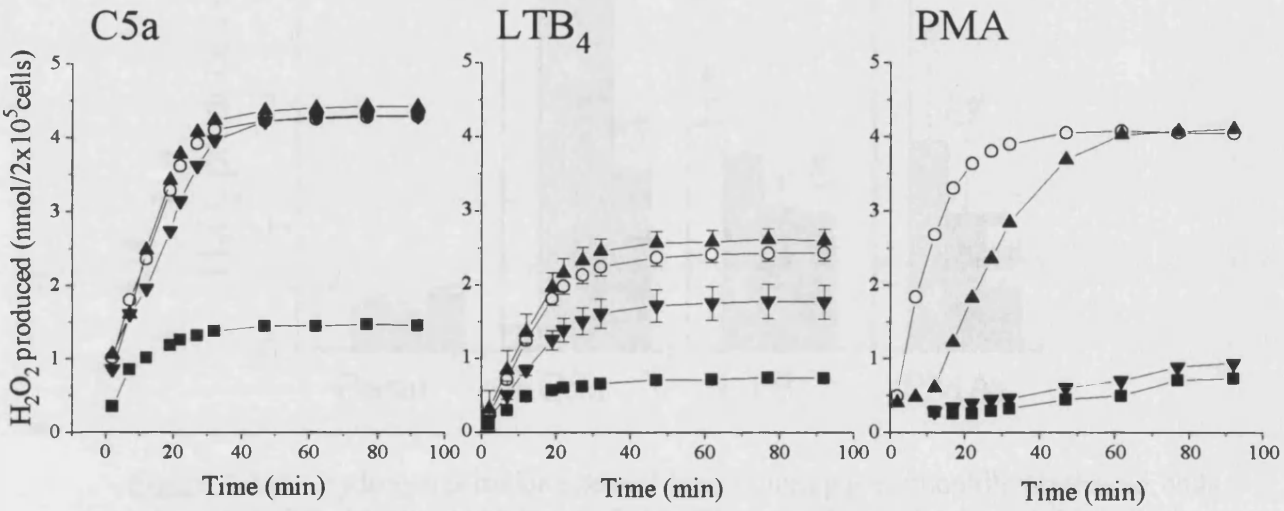


Figure 3.12 Effect of Ro 31-8220/002 on guinea pig eosinophil H_2O_2 production. Eosinophils were pretreated with vehicle (○), 300nM (▲), 1μM (▼) or 3μM (■) Ro 31-8220/002 for 20min at 37°C before stimulation with C5a (30nM), LTB_4 (30nM) or PMA (300pM). Representative of 5-6 experiments.

The effect of Ro 31-8220/002 was not due to cell cytotoxicity as eosinophils at the end of the experiment were >99% viable as assessed by trypan blue exclusion. The results are summerized in figure 3.13. These experiments were also carried out in the stirred cell system, and a similar inhibition to that seen in the microassay with Ro 31-8220/002 (3μM) was observed (figure 3.14).

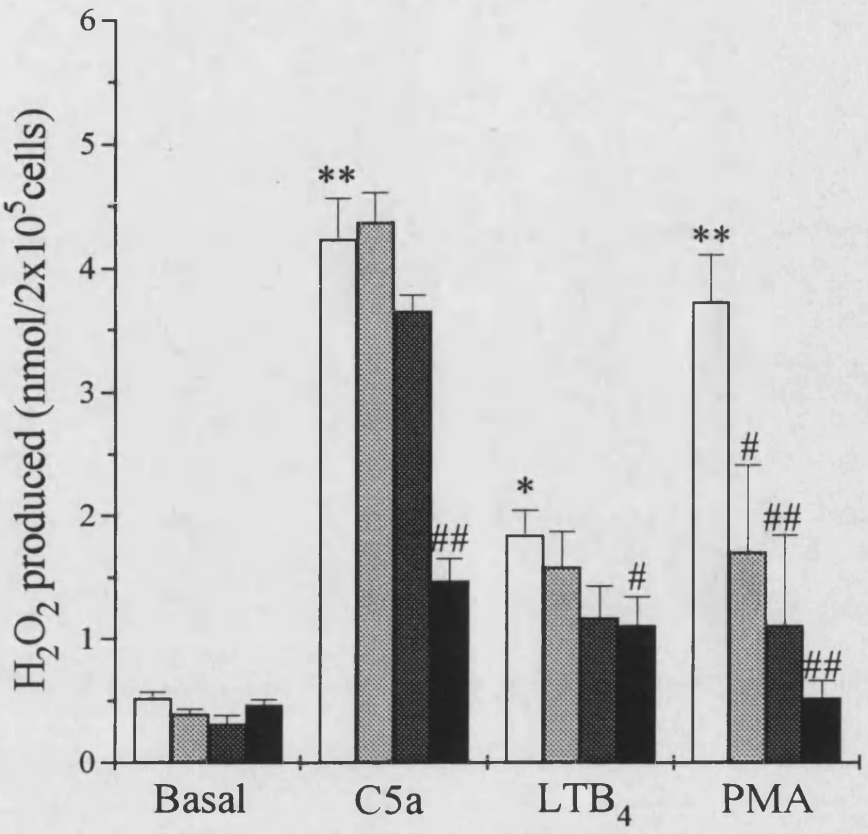


Figure 3.13 Hydrogen peroxide released from guinea pig eosinophils pretreated with vehicle (□), 300nM (▒), 1μM (▩) or 3μM (■) of the PKC selective inhibitor Ro 31-8220/002 (for 20min at 37°C), either unstimulated or stimulated with C5a (30nM), LTB_4 (30nM) or PMA (300pM) at t=30min. Bars indicate mean±sem nmol H_2O_2 produced /2x10^5 cells for n=5-6 different eosinophil preparations. Significantly increased H_2O_2 released compared with basal H_2O_2 produced: *, $p<0.05$; **, $p<0.01$. Significantly reduced H_2O_2 produced compared with vehicle treated cells: #, $p<0.05$; ##, $p<0.01$.

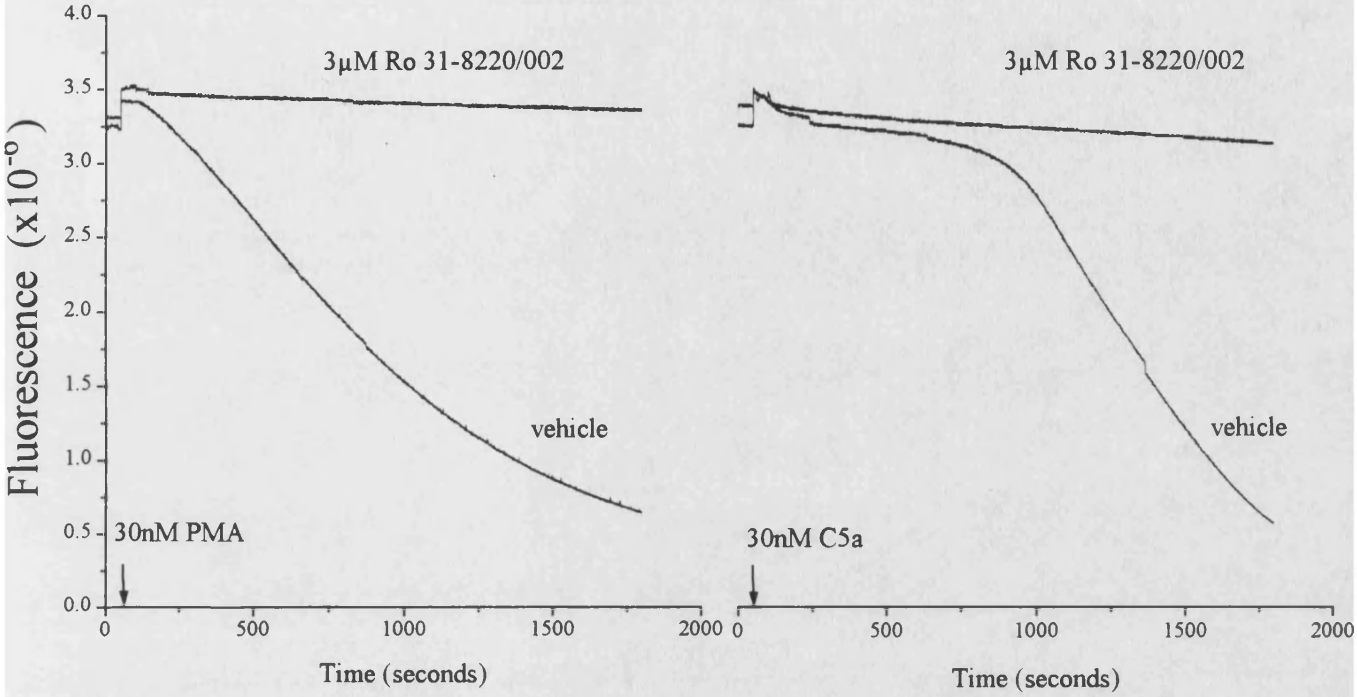


Figure 3.14 Hydrogen peroxide released from guinea pig eosinophils in stirred suspension pretreated with Ro 31-8220/002 (3μM) for 20min at 37°C before stimulation with PMA (30nM) or C5a (30nM). Traces are representative of 2 different eosinophil preparations.

3.3.6 Effect of the tyrosine kinase inhibitors genistein, erbstatin, herbimycin A and tyrphostin-A47

Other signalling pathways may also exist, as 100% inhibition was not obtained by the PKC inhibitor Ro 31-8220/002. To investigate whether a tyrosine kinase mechanism was involved in guinea pig eosinophils, the specfic tyrosine kinase inhibitors genistein, erbstatin, herbimycin A and tyrphostin-A47 were used. Because these inhibitors were fluorescent, the effect of each inhibitor was first investigated to see if it had any effect on the H_2O_2 assay. Figure 3.15 shows that erbstatin (10μM), tyrphostin-A47 (100μM) and high concentrations of genistein (300μM) interfered with the assay.

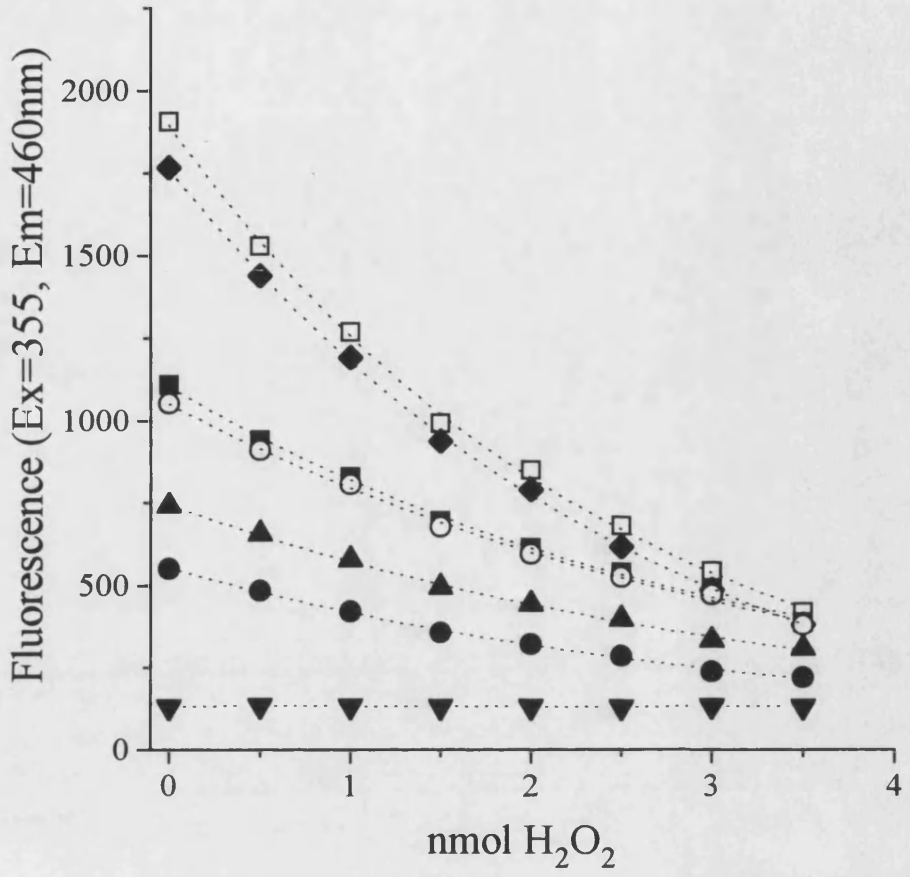


Figure 3.15 Effect of the fluorescent properties of different specific tyrosine kinase inhibitors on the scopoletin based hydrogen peroxide assay: vehicle (□); herbimycin A (1μM)(◆); tyrphostin A-47 (10μM)(■), (100μM)(▲); genistein (100μM)(○), (300μM)(●) and erbstatin (10μM)(▼).

The effect of herbimycin A (1μM) and genistein (100μM) was thus investigated on H_2O_2 production. After incubation for 2½ and 4 hours (as shorter incubation times had no effect) with the respective tyrosine kinase inhibitors, the guinea pig eosinophils were plated out and the agonists C5a, PAF (30-100nM) and PMA (3nM) were added. As figure 3.16 shows, the time course of H_2O_2 production was similar to that of stimulated guinea pig eosinophils not incubated with the tyrosine kinase inhibitors. However due to the long incubations, some sensitivity to the agonists had been lost and

the overall H_2O_2 released was lower.

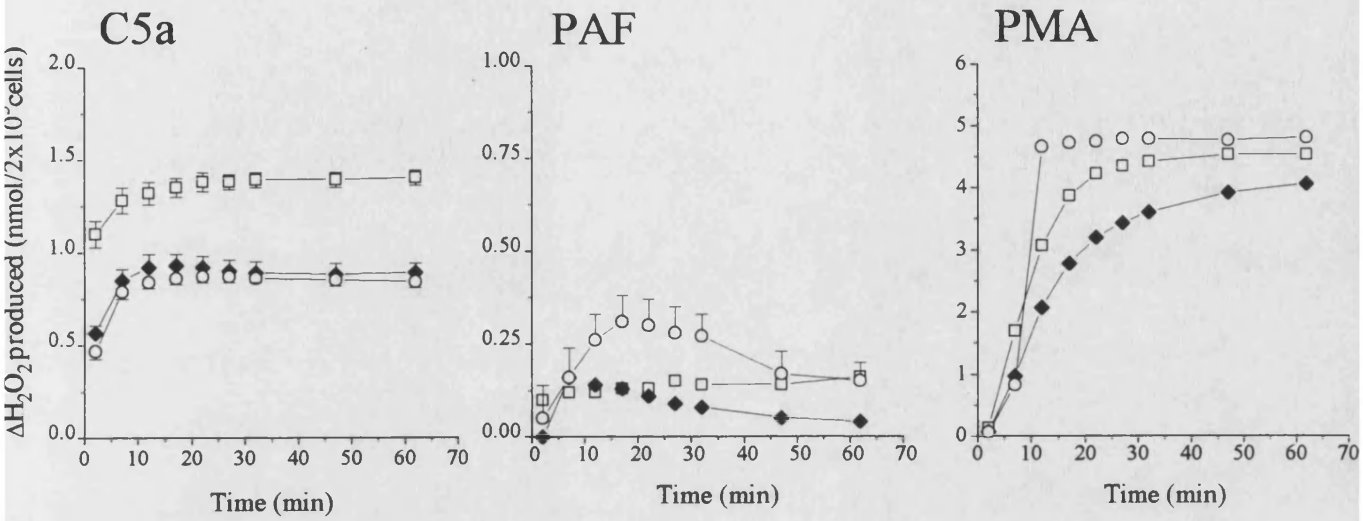


Figure 3.16 Effect of specific tyrosine kinase inhibitors on guinea pig eosinophil H_2O_2 production. Eosinophils were pretreated with vehicle (□), hebimycin A (1μM)(◆) or genistein (100μM)(○) for 2½ hours at 37°C before stimulation with C5a (100nM), PAF(100nM) or PMA (3nM). Traces show change of H_2O_2 release above basal levels. Representative of 2-5 experiments.

Very little H_2O_2 was detected from cells that had been incubated for 4 hours (data not shown). Both herbimycin A (1μM) and genistein (100μM) inhibited the production of H_2O_2 elicited by C5a (100nM) in guinea pig eosinophils, though this did not reach significance. Only herbimycin A appeared to have an effect on PMA (3nM) stimulated cells. Figure 3.17 shows the small inhibition of H_2O_2 release caused by the pretreatment of eosinophils with hebimycin A (1μM). The cells were still >99% viable as assessed by trypan blue exclusion.

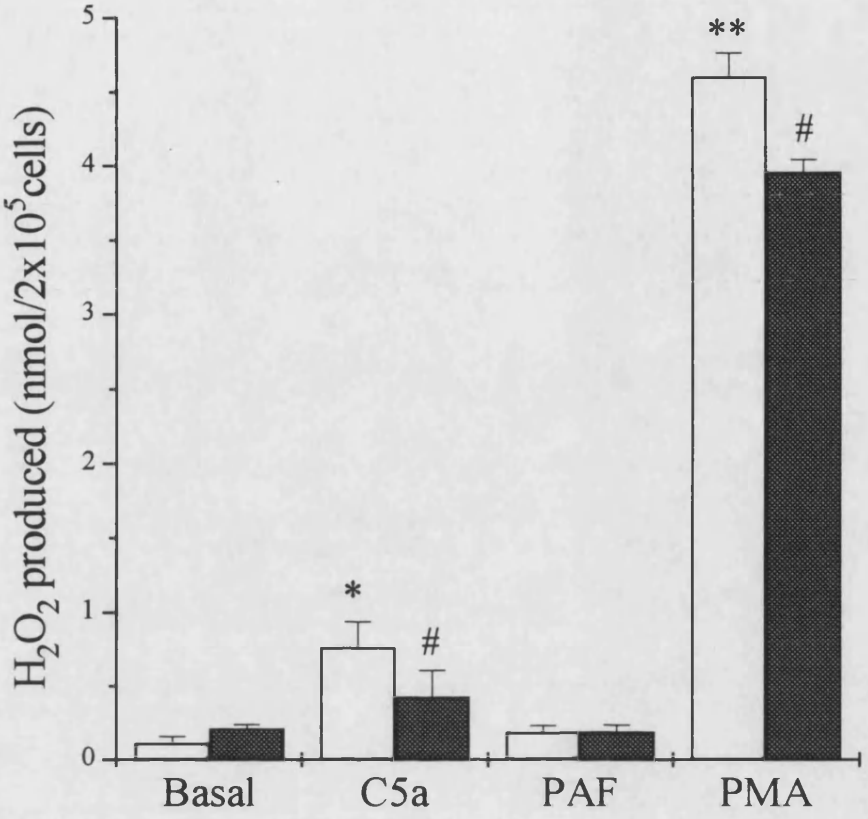


Figure 3.17 Hydrogen peroxide released from guinea pig eosinophils pretreated with vehicle (□) or the selective tyrosin kinase inhibitor herbimycin A (1μM)(▩) (for 2½ hours at 37°C) then stimulated with C5a (100nM), PAF (100nM) or PMA (3nM) at t=30min. Bars indicate mean±sem nmol H_2O_2 produced/2x10^5 cells for n=3-5 different eosinophil preparations. Significantly increased H_2O_2 released compared with basal H_2O_2 produced: *, $p<0.05$; **, $p<0.01$. Significantly reduced H_2O_2 produced compared vehicle treated cells: #, $p<0.05$.

3.3.7 Effect of wortmannin on H_2O_2 release

To ascertain whether a role for PI-3 kinase exists in guinea pig eosinophils, the specific PI 3-kinase inhibitor wortmannin was used together with C5a (100nM) and PAF (100nM). Figure 3.18 shows that wortmannin (3-30nM) inhibited H_2O_2 production elicited by C5a stimulated guinea pig eosinophils (significant at 30nM). Again the effect of wortmannin was not due to cell cytotoxicity as eosinophils at the

end of the experiment were >99% viable as assessed by trypan blue exclusion.

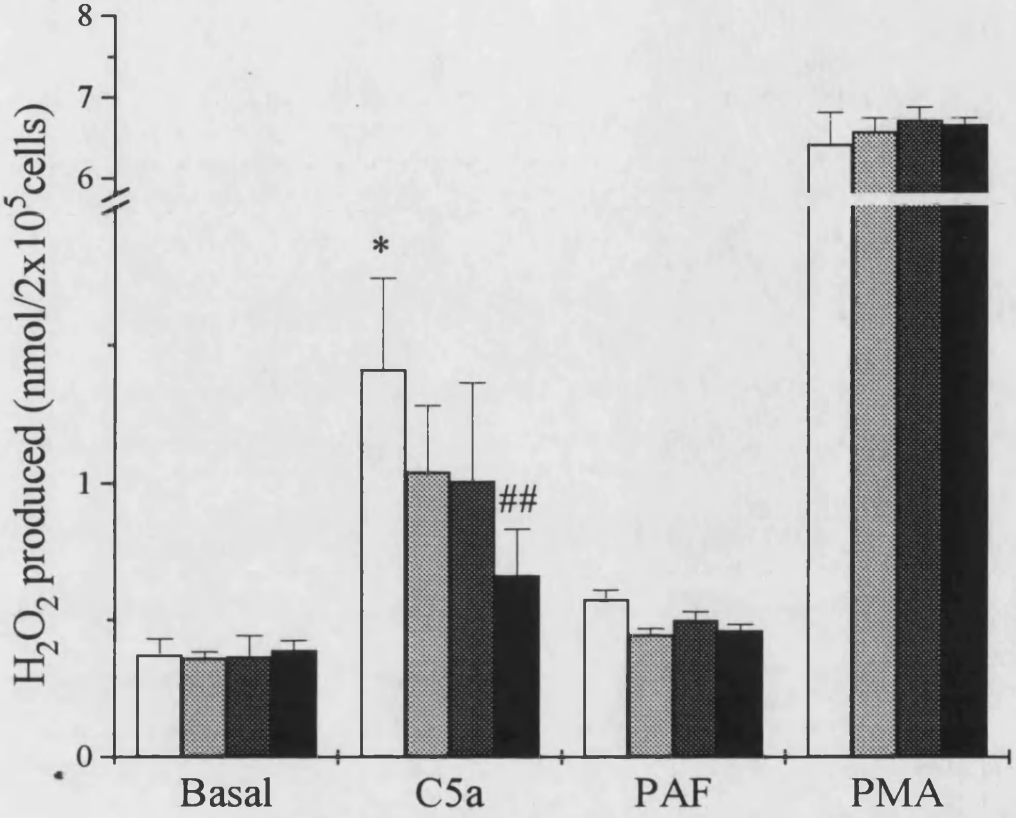


Figure 3.18 Hydrogen peroxide released from guinea pig eosinophils pretreated with vehicle (□), 3nM (▒), 10nM (▩) or 30nM (■) of the PI-3 kinase inhibitor wortmannin (for 10min at 37°C), then stimulated with either C5a (100nM), PAF (100nM) or PMA (1nM) at t=30min. Bars indicate mean±sem nmol H_2O_2 produced/2x10^5 cells for n=3 different eosinophil preparartions. Significantly increased H_2O_2 released compared with basal H_2O_2 produced: *, $p<0.05$. Significantly reduced H_2O_2 produced compared with vehicle treated cells: ##, $p<0.01$ (2 way-ANOVA only).

3.3.8 Effect of priming guinea pig eosinophils

To establish whether the activity of guinea pig eosinophils could be enhanced, they were incubated with the cytokine GM-CSF (100pM) for either 10 min or 1 hour before stimulation with a variety of agonists. A 10 min preincubation had no effect on enhancing H_2O_2 production. Though a 1 hour preincubation with GM-CSF was able

to augment H_2O_2 release from C5a (10-100nM)-stimulated cells above vehicle treated cells (figure 3.19), this effect is unlikely to be due to cellular priming. PAF (10pM-1μM), IL-8 (1-10nM) or NAP-2 (1-100nM) treated cells which were poor activators of H_2O_2 production had no enhanced activity when incubated with GM-CSF.

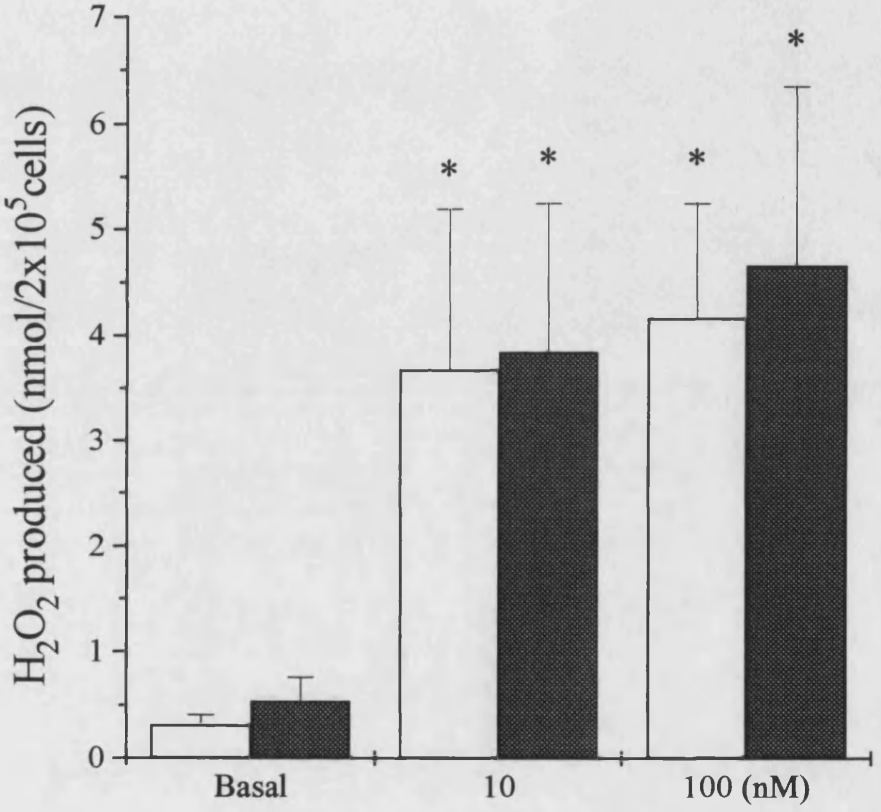


Figure 3.19 Hydrogen peroxide released from guinea pig eosinophils pretreated with vehicle (□) or GM-CSF (100pM)(▩) (for 1 hour at 37°C) then stimulated with C5a (10-100nM) at t=30min. Bars indicate mean±sem nmol H_2O_2 produced/2x10^5 cells for n=3-4 different eosinophil preparations. Significantly increased H_2O_2 released compared with respective basal H_2O_2 produced: *, $p<0.05$.

3.3.9 Effect of agonists on BAL eosinophils

The effect on H_2O_2 production elicited by various agonists was compared on eosinophils obtained from BAL to those elicited into the peritoneal cavity. The amount

of H_2O_2 released from BAL eosinophils by activating them with C5a, PAF (30-100nM) and PMA (3nM) was measured in eosinophils obtained from guinea pigs that had been sensitised and then challenged to ovalbumin (OA-challenged), challenged to saline (sham-challenged) or those that had not been challenged (non-challenged).

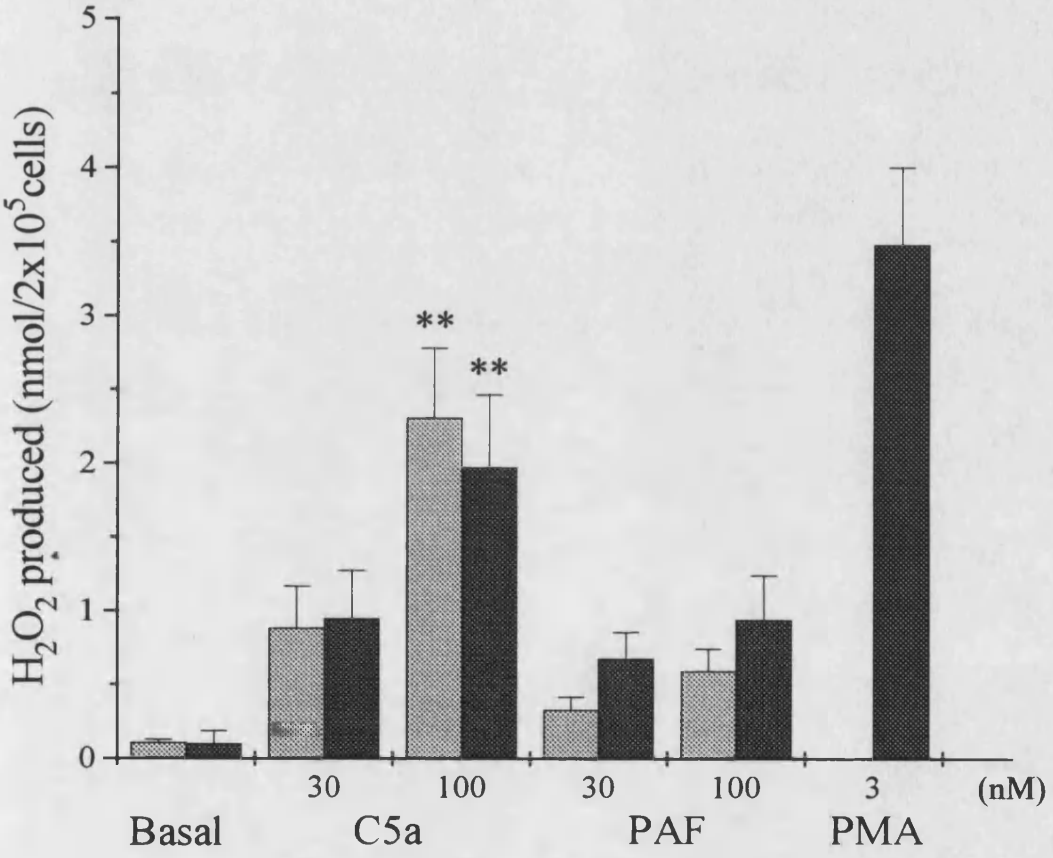


Figure 3.20 Hydrogen peroxide released from guinea pig BAL eosinophils: non-/sham-challenged animals (▒) or OA-challenged animals (▓); either unstimulated, or stimulated with C5a (30-100nM), PAF (30-100nM) or PMA (3nM)at t=30min. Bars indicate mean±sem nmol H_2O_2 produced/2x10^5 cells for n=3-6 different eosinophil preparations. Significantly increased H_2O_2 released compared with respective basal H_2O_2 produced: **, $p<0.01$.

The number of eosinophils obtained from BAL's were very low, especially in the case of non-challenged animals, thus the data from non-challenged and sham-challeged animals were pooled. C5a (30nM and 100nM) and PMA (3nM) still elicited

good amounts of H_2O_2 from BAL eosinophils from each source. PAF (30nM and 100nM) showed a lower amount of H_2O_2 production from eosinophils obtained from non-/sham-challenged animals, but an enhancement of H_2O_2 (though not significant) released from eosinophils of OA-challenged animals compared to that of non-/sham-challenged animals (figure 3.20).

3.4 Human eosinophils

3.4.1 Effect of added agonists, including C-C chemokines and cytokines

C5a (1-100nM), PAF (1-100nM) and both the C-C chemokines RANTES (10-100nM) and MCP-3 (1-100nM) could stimulate H_2O_2 production from human eosinophils, causing a dose related release of H_2O_2 that was significant at 10nM and 100nM (figures 3.21 and 3.22) for all agonists compared with unstimulated cells. Also the cytokine IL-5 was an extremely potent stimulator of H_2O_2 production by human eosinophils, again causing a dose-related release of H_2O_2 that was significant at 10pM and above (figure 3.22). Figure 3.21 shows that nearly all the H_2O_2 was released within the first 30min in C5a, PAF, RANTES and MCP-3 stimulated cells and within the first 60min for IL-5 stimulated cells. All the initial rates of reaction were comparable for the different agonists, without a negligible lag between the addition of agonist and start of H_2O_2 production.

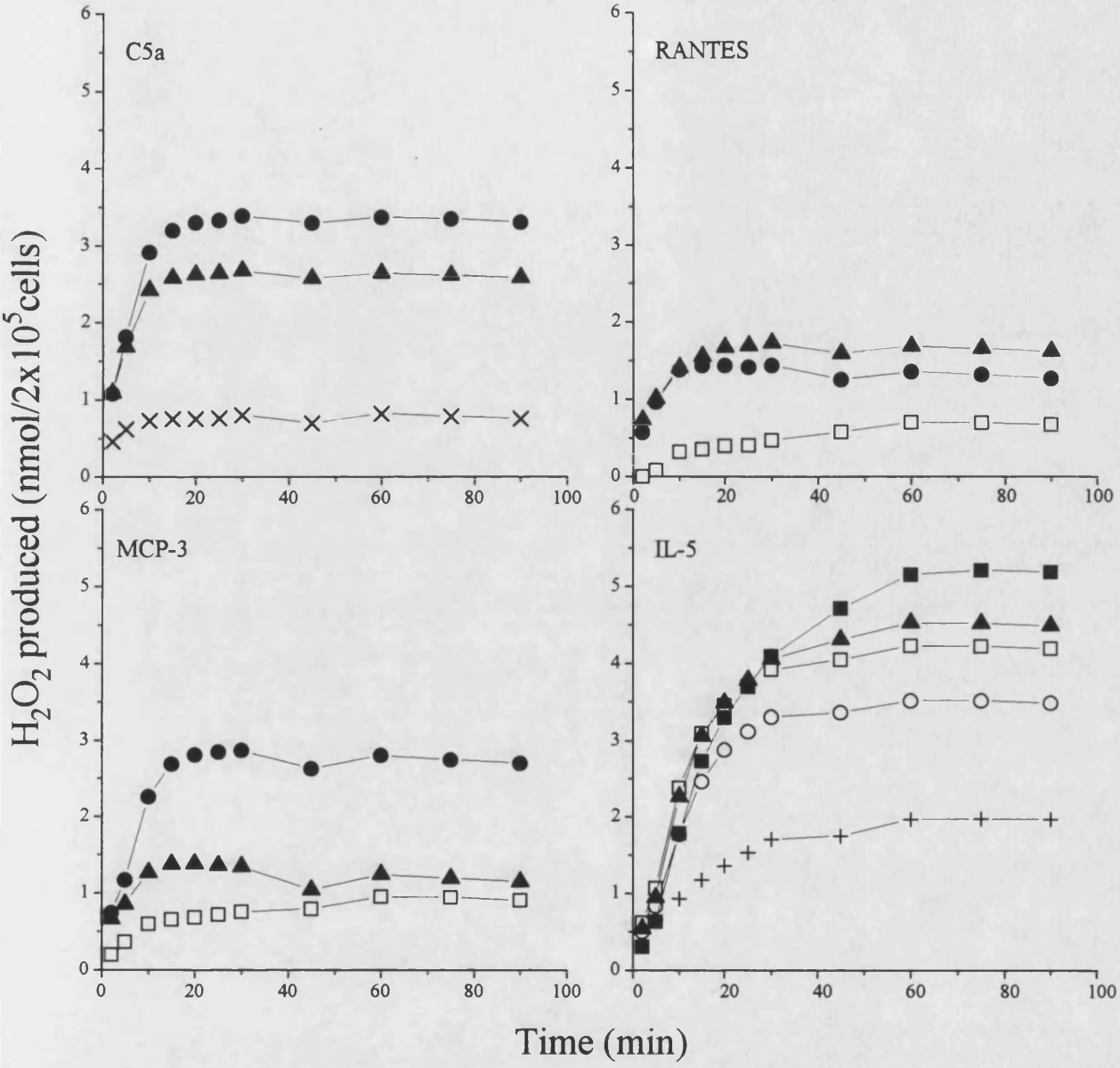


Figure 3.21 Time course of hydrogen peroxide production from human eosinophils stimulated with different concentrations of C5a, RANTES, MCP-3 and IL-5. Traces indicate mean±sem nmol H_2O_2 production/2x10^5 cells from triplicate wells. Doses: vehicle (x); 10pM (+); 100pM (○); 1nM (□); 10nM (▲); 40nM (■) 100nM (●). Representative of 3-20 experiments.

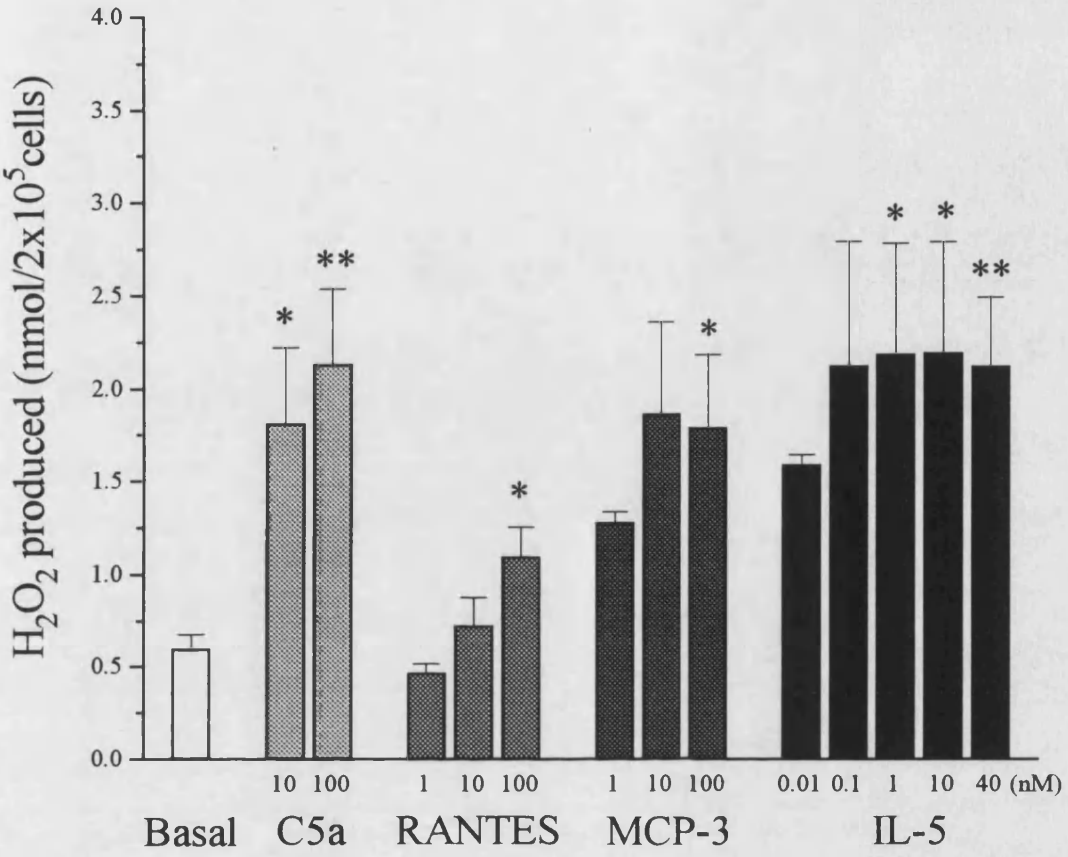


Figure 3.22 Hydrogen peroxide released from human eosinophils either unstimulated (□) or stimulated with C5a (▒), RANTES (▨), MCP-3 (▩) or IL-5 (■) at t=30min. Bars indicate mean±sem nmol H_2O_2 produced/ $2x10^5$ cells for n=3-20 different eosinophil preparations. Significantly increased H_2O_2 produced compared with vehicle treated cells: *, $p<0.05$; **, $p<0.01$.

The cytokines MCP-1, MIP-1α and MIP-1β were also tested and were inactive in this assay (table 3.3), with C5a (100nM) being a positive control. IL-8 also activated human eosinophils to release H_2O_2.

Table 3.3 Effects of MIP-1α, MIP-1β, IL-8, MCP-1 and PAF on H_2O_2 production from human eosinophils at t=30min. Data indicates mean±sem nmol H_2O_2 produced/2x10^{5}cells for triplicate wells. Representative of 3-5 experiments.

Agonist	H_2O_2 produced (nmol/2x10^{5}cells) (t=30min)
Basal	0.491±0.02
100nM MIP-1α	0.349±0.04
100nM MIP-1β	0.413±0.04
100nM IL-8	0.723±0.05
100nM PAF	0.789±0.01
100nM MCP-1	0.317±0.03
100nM C5a	1.23±0.01

3.4.2 Effect of priming human eosinophils with IL-5

The ability of IL-5 to upregulate human eosinophils was also tested. Human eosinophils were co-incubated with different concentrations of RANTES (10-100nM) and IL-5 (0.4-40nM) and monitored for H_2O_2 release for up to 2 hours at 37°C. Figure 3.23 shows that the combined effects of RANTES and IL-5 were additive and not synergistic (due to sample variance, these increases were not significant).

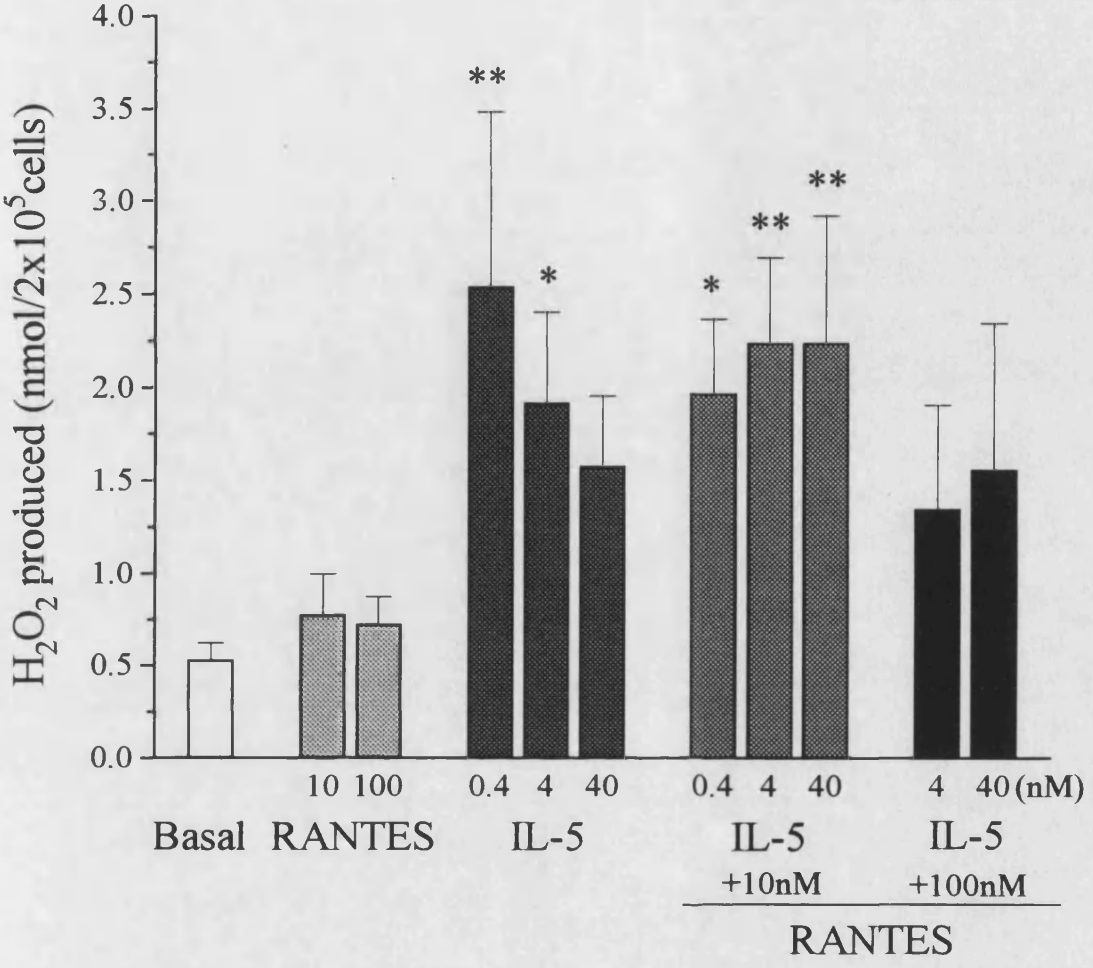


Figure 3.23 Hydrogen peroxide released from human eosinophils in response to RANTES (10-100nM)(▒), IL-5 (0.4-40nM)(▓), IL-5 (0.4-40nM) plus RANTES (10nM)(▒) or IL-5 (4-40nM) plus RANTES (100nM)(■) at t=60min. Bars indicate the mean±sem nmol H_2O_2 produced/2x10^5 cells for n=3 different eosinophils. Significantly increased H_2O_2 released compared with basal H_2O_2 produced: *, $p<0.05$; **, $p<0.01$.

3.4.3 Effect of the ROCC blocker, SK&F 96365, on H_2O_2 production on human eosinophils

On guinea pig eosinophils, SK&F 96365 appeared to have an agonistic effect on H_2O_2 release rather than an inhibitory effect, thus the compond was tested to see if it exerted similar properties on human eosinophils. Interestingly, the opposite was

true, SK&F 96365 (3-60μM) had no effect on H_2O_2 release from human eosinophils (see table 3.4) and preliminary experiments suggested that H_2O_2 released from C5a (100nM) and RANTES (100nM) stimulated cells could be reduced by SK&F 96365 (data not shown).

Table 3.4 Effect of the ROCC blocker, SK&F 96365, on H_2O_2 production from human eosinophils at t=30min. Data indicates mean±sem nmol H_2O_2 produced/2x10^5cells from n=3 different eosinophil preparations.

Agonist	H_2O_2 produced (nmol/2x10^5 cells) (t=30 min, n=3)
Basal	0.418 ± 0.065
3μM SK&F 96365	0.437 ± 0.112
10μM SK&F 96365	0.342 ± 0.083
30μM SK&F 96365	0.225 ± 0.086
60μM SK&F 96365	0.168 ± 0.071
100nM C5a	1.520 ± 0.466

3.4.4 Inhibition of H_2O_2 with the selective PKC inhibitor, Ro 31-8220/002

As for guinea pig eosinophils, human eosinophils were investigated for different signalling mechanisms. Again the selective protein kinase C inhibitor Ro 31-8220/002 was used in conjunction with with C5a, RANTES, MCP-3, IL-5 and PMA. Figure 3.24 shows that Ro 31-8220/002 could dose-dependently inhibit H_2O_2 release from human eosinophils activated by each agonist. Ro 31-8220/002 (3μM) caused at least 80% inhibition for each agent tested.

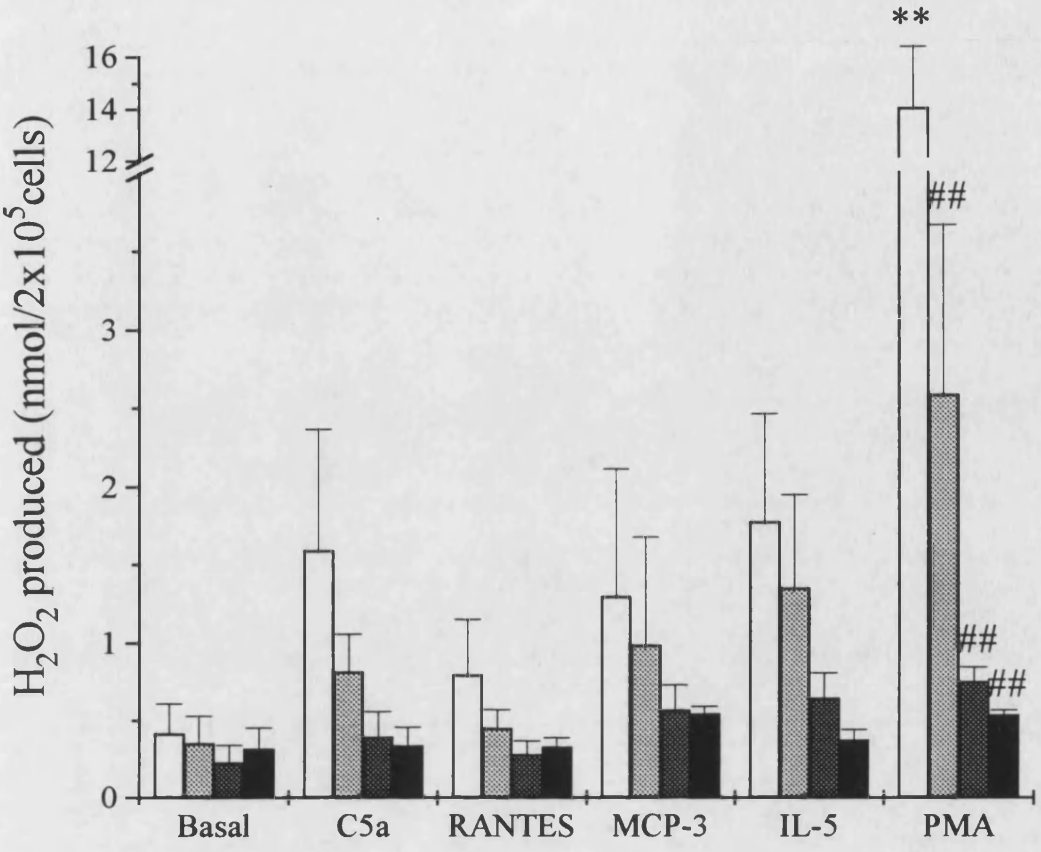


Figure 3.24 Hydrogen peroxide released from human eosinophils pretreated with vehicle (□), 300nM (▒), 1μM (▓) or 3μM (■) of the PKC selective inhibitor Ro 31-8220/002 (for 20min at 37°C) in either unstimulated or C5a (100nM), RANTES (100nM), MCP-3 (100nM), IL-5 (40nM) or PMA (1nM) stimulated cells at t=30min. Bars indicate mean±sem nmol H_2O_2 produced/2x10^5 cells for n=3 different eosinophil preparations. Significantly increased H_2O_2 released compared with basal H_2O_2 produced: **, $p<0.01$. Significantly reduced H_2O_2 produced compared with vehicle treated cells: ##, $p<0.01$.

3.4.5 Inhibition of H_2O_2 with the selective PI-3 kinase Inhibitor, wortmannin

Similarly, to investigate whether a role for PI-3 kinase existed in the signalling pathway of H_2O_2 production in human eosinophils, the selective PI-3 kinase inhibitor, wortmannin, was used together with C5a, RANTES, MCP-3, IL-5 and PMA. Figure 3.25 shows, wortmannin (3-30nM) dose dependently-inhibited unstimulated, C5a, RANTES, MCP-3 and IL-5 stimulated eosinophils. Figure 3.26 summarises the pooled data and shows that this inhibition was significant for RANTES, MCP-3 and IL-5 stimulated eosinophils at 3nM, 10nM and 30nM of wortmannin.

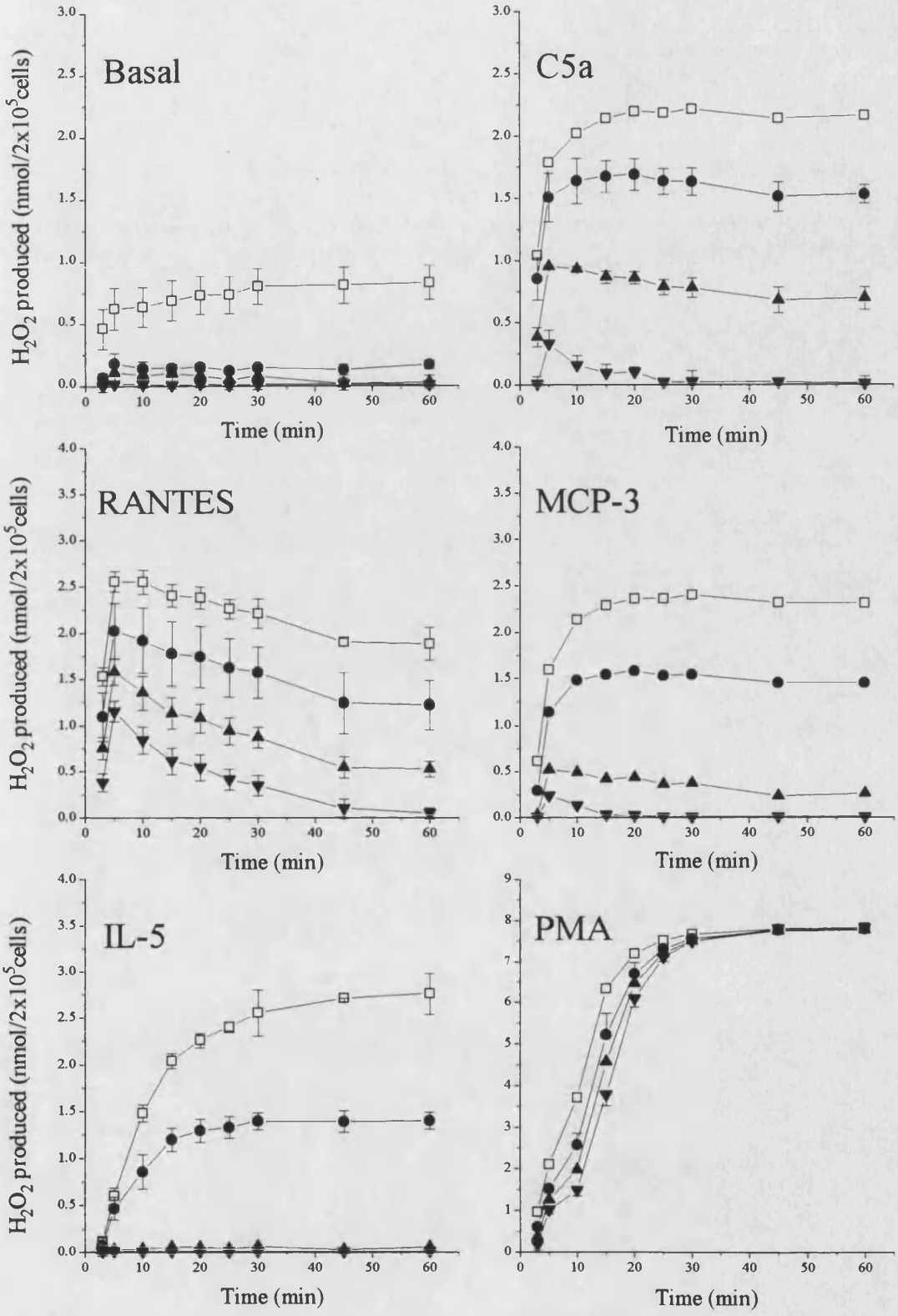


Figure 3.25 Effect of wortmannin on time courses of H_2O_2 production from human eosinophils. Eosinophils were pretreated with vehicle (□), 3nM (●), 10nM (▲) or 30nM (▼) wortmannin for 10min at 37°C before stimulation with C5a (100nM), RANTES (100nM), MCP-3 (100nM), IL-5 (40nM) or PMA (1nM). Traces indicate mean±sem nmol H_2O_2 production from triplicate wells. Representative of 3-7 experiments.

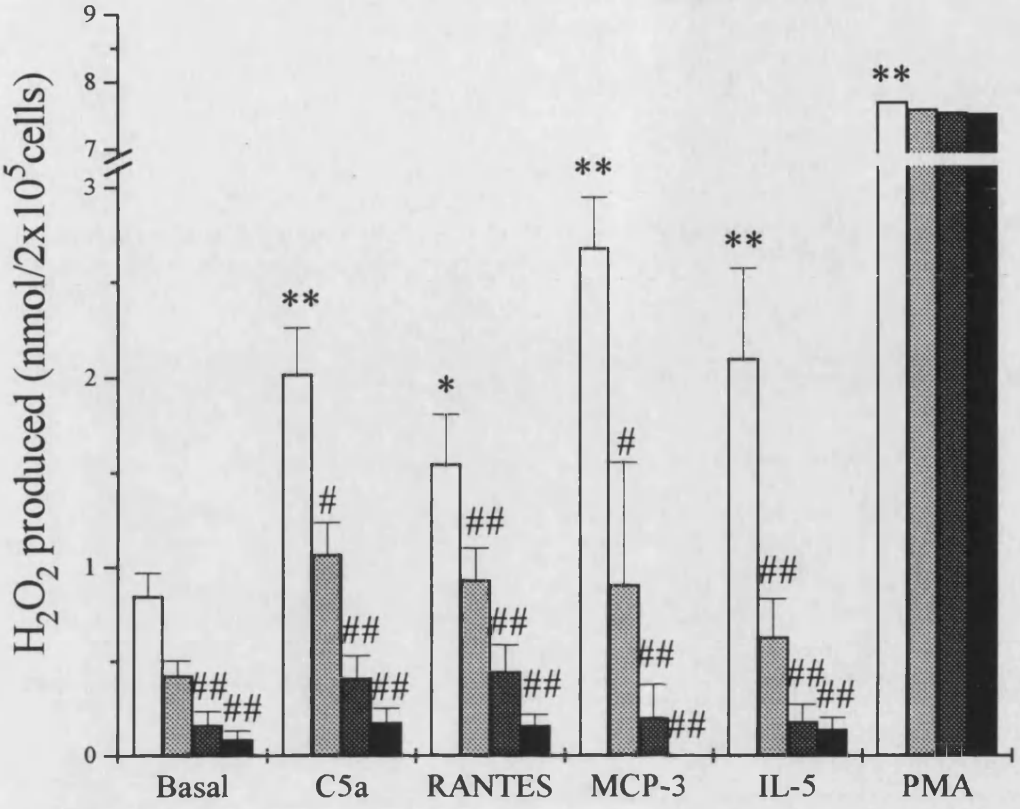


Figure 3.26 Hydrogen peroxide released from human eosinophils pretreated with vehicle (□) , 3nM (▒), 10nM (▓) or 30nM (■) of the PI 3-kinase inhibitor wortmannin(for 10min at 37°C) then stimulated with either C5a (100nM), RANTES (100nM), MCP-3 (100nM), IL-5 (40nM) or PMA (1nM) at t=30min. Bars indicate mean± sem nmol H_2O_2 produced/2x10^5 cells for n=3-7 different eosinophil preparartions. Significantly increased H_2O_2 released compared with basal H_2O_2 produced: **, $p<0.01$. Significantly reduced H_2O_2 produced compared with vehicle treated cells: #, $p<0.05$; ##, $p<0.01$.

Wortmannin was unable to inhibit PMA (1nM) which is an extremely rapid activator of H_2O_2 production via a protein kinase C mechanism, indicating wortmannin was not affecting the PKC signalling mechanism. The effect of wortmannin was not due to cytotoxicity either, as human eosinophils at the end of the experiment were 99% viable assessed by trypan blue exclusion.

3.5 Eol-1 and Eol-3 responses to added agonists

Eol-1s and Eol-3s were very poor producers of H_2O_2. C5a (100nM), PAF (100nM), various cytokines including MCP-1 (100nM) and RANTES (100nM) and even PMA (3-30nM) were tested for their ability to cause H_2O_2 release from Eol's. Unmatured Eol-1s, unmatured Eol-3s, butyric acid (0.5mM) four-day matured Eol-1s or butyric acid (0.5mM) four-day matured Eol-3s were all tested for H_2O_2 release. All were unable to produce measurable amounts of H_2O_2.

3.6 Control experiments

3.6.1 Cytochrome c experiments

To confirm that the superoxide ion was leading to the formation of H_2O_2, several cytochrome *c* experiments were carried out. These experiments demonstrated that similar results could be obtained via the superoxide dismutase-inhibitable reduction of cytochrome *c* as in the scopoletin-based assay. Figure 3.27 demonstrates that the same order of potency could be achieved using the cytochrome *c* assay with guinea pig eosinophils and added agonist, *viz.* PMA > C5a > PAF > basal.

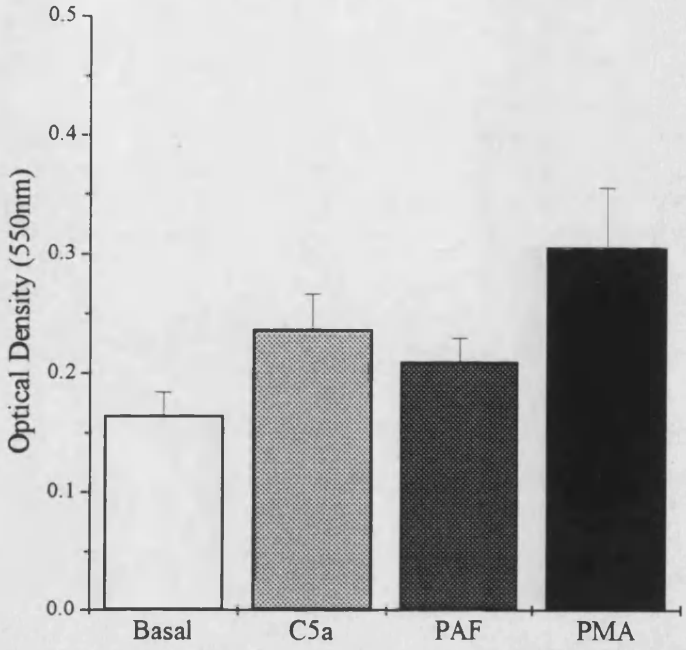


Figure 3.27 Superoxide release from guinea pig eosinophils either unstimulated (□) or stimulated with C5a (100nM)(▒), PAF (100nM)(▓) or PMA (1nM)(■) at t=15min. Bars indicate mean±sem increase in absorbance at 550nm of agonist compared to agonist with SOD (100U/ml) for quadruplicate wells. Representative of 3 experiments.

3.6.2 Effect of catalase and HMAP on H_2O_2 detection

To inhibit the H_2O_2 assay catalase prevents horseradish peroxidase reducing the scopoletin fluoresence by breaking down H_2O_2. Figure 3.28A shows that H_2O_2 can be inhibited by 70% and 75% with catalase (3000U/ml) for C5a (100nM) and LTB_4 (100nM) respectively.

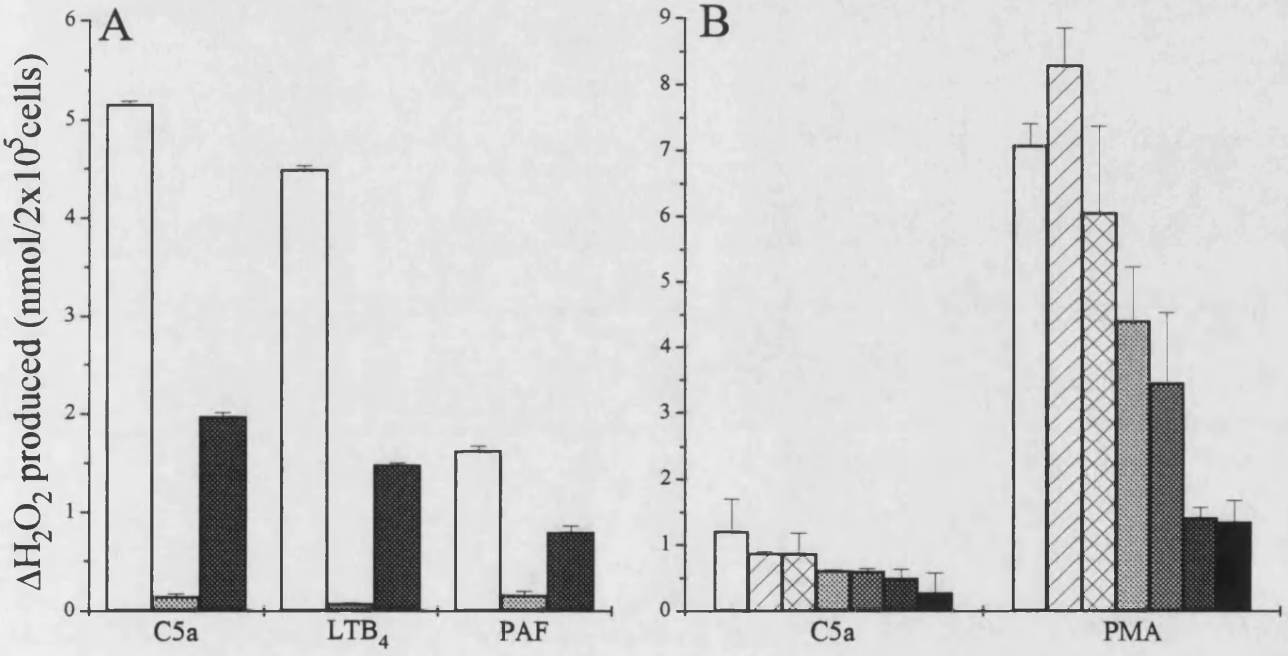


Figure 3.28 (A) Hydrogen peroxide released from guinea pig eosinophils stimulated with C5a (100nM), LTB_4 (100nM) or PAF (100nM) in the presence of vehicle (□), without HPO (▒) or with HPO and catalase (3000U/ml)(■) at t=60min. (B) Hydrogen peroxide released from human eosinophils stimulated with C5a (100nM) or PMA (1nM) in the presence of vehicle (□) or HMAP; 3μM (▨), 10μM (⊠), 30μM (▒), 100μM (▩), 300μM (▩) or 1mM (■) at t=60min. Bars indicate mean±sem ΔH_2O_2 nmol produced/2x10^{5}cells above basal levels from triplicate wells. Representative of 2-3 experiments.

Two NADPH oxidase inhibitors were investigated for their effect on H_2O_2 production from eosinophils. DPI and HMAP were highly fluorescent compounds, thus their effect on the standard calibration curve was also measured (data not shown). DPI was very insoluble (and expensive) so no H_2O_2 assay was successfully carried out

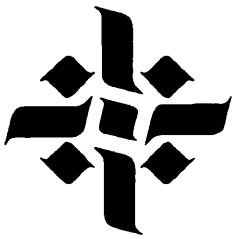
in the presence or absence of added agonists. HMAP (3μM-1mM) was used effectively and found to dose-dependently inhibit H_2O_2 release from human eosinophils (figure 3.28B). This confirmed that the H_2O_2 being measured by the scopoletin-based assay was produced via the NADPH oxidase complex.

3.7 Summary of H_2O_2 experiments

- Guinea pig and human eosinophils can be stimulated to produce H_2O_2 with various soluble stimuli.
- Order of potency for H_2O_2 production from guinea pig eosinophils: C5a > LTB_4 > PAF.
- Order of potency for H_2O_2 production from human eosinophils: IL-5 > MCP-3 > C5a > RANTES > PAF.
- Responses of human eosinophils could be enhanced by IL-5. GM-CSF failed to significantly increase guinea pig eosinophil responses.
- Guinea pig eosinophils obtained from BAL rather than the peritoneal cavity, could also be made to release H_2O_2. Eosinophil activity from challenged animals appeared to be upregulated.
- Extracellular calcium and magnesium were required for H_2O_2 release. This was demonstrated with the chelators EGTA and EDTA. Some extracellular calcium may be influxing into the cell as shown by the $NiCl_2$ experiments in stirred cells, however the amount of calcium in intracellular stores may be sufficient to elicit a response.
- The ROCC blocker SK&F 96365 appeared to be an activator of H_2O_2

production in guinea pig eosinophils but not in human eosinophils.

- Guniea pig eosinophils were sensitive to the tyrosine kinase inhibitor, herbimycin A.
- Both guinea pig and human eosinophil H_2O_2 release by added agonist could be inhibited by micromolar conctrations of the selective PKC inhibitor, Ro 31-8220/002.
- Both guinea pig and human eosinophil H_2O_2 release by added agonist could be inhibited by nanomolar concentrations of the specific PI-3 kinase inhibitor, wortmannin.
- The eosinophilic cell lines Eol-1 and Eol-3 were unable to release H_2O_2 when stimulated with various agonists.



4 Eosinophil Adhesion

4.1 Rose bengal adhesion assay

When setting up the H_2O_2 assay (see section 3.2), the choice of plates and protein coating and the subsequent effect they may have had on the adhesion assay was also considered. Clearly any plate with a particular protein coating that caused spontaneous H_2O_2 release was discounted as the effects between eosinophil H_2O_2 production and adhesion were being assessed. Therefore, uncoated plates, ELISA plates and the coatings BSA, FCS, fibronectin and gelatin were not considered. Figure 4.1 shows that fibrinogen (10mg/ml) and laminin (3μg/ml) allowed different magnitudes of eosinophil adhesion to various added agonists. The window of detection was wider in flexipates when comparing vehicle treated cells to C5a stimulated cells as opposed to the titertek plates.

Though fibrinogen and laminin gave similar responses of changes in eosinophil adhesion to added agonist, fibrinogen had the capacity to avidly bind the rose bengal stain in wells where no cells were present. This in some circumstances could of made it difficult to detect whether any eosinophils had adhered to the plate at all. Laminin appeared to be the best protein coating in the H_2O_2 assay and the preferred protein coating for the adhesion assay. In conclusion laminin-coated microtitre flexiplates gave the best overall results in both the H_2O_2 and adhesion assays.

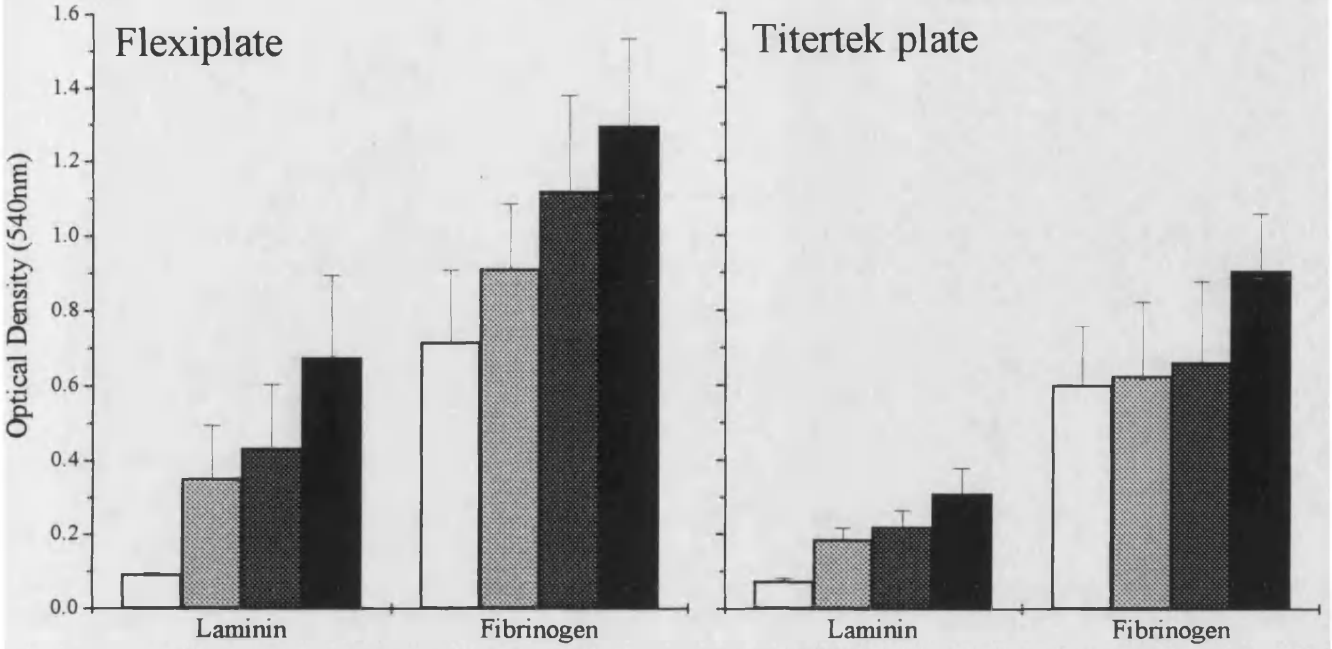


Figure 4.1 Adhesion of guinea pig eosinophils in either flexiplates or titertek plates. Wells were left blank (□) or contained eosinophils that were either unstimulated (▒) or stimulated with C5a (100nM)(▩) or PMA (100nM)(■) in laminin-coated (3μg/ml) or fibrinogen-coated (10mg/ml) microtitre plates at t=30min. Bars indicate optical density of eosinophil rose bengal uptake at 540nm for n=3 experiments.

4.2 Guinea pig eosinophils

4.2.1 Effect of added agonist on adhesion

To assess whether adhesion was a requisite or an enhancer for H_2O_2 production, after each assay the non-adherent cells were removed and adhesion quantified by rose bengal stain. Figure 4.2 shows a representative experiment of calibrating the number of cells with rose bengal uptake. This was a reproducible linear relationship. A linear regression fit gave the parameters shown in table 4.1.

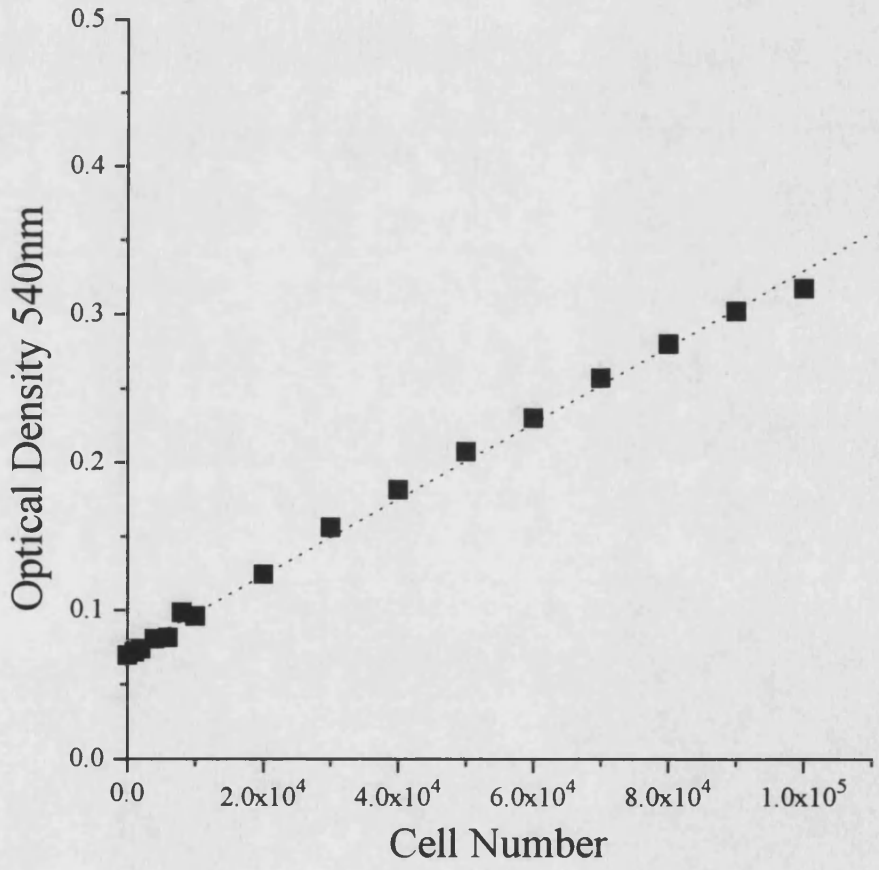


Figure 4.2 Standard curve for number of guinea pig eosinophils with rose bengal uptake. Trace indicates optical density measured at 540nm from triplicate wells. Representative of 2 experiments.

Using the equation 2.7 the number of cells adhered to the laminin-coated

microtitre plate was calculated for all subsequent experiments.

Table 4.1 Parameters of linear regression for figure 4.2

Parameter	Value	SD
A	0.7225	0.002
B	2.5721×10^{-6}	4.0685×10^{-8}
R	0.99825	-

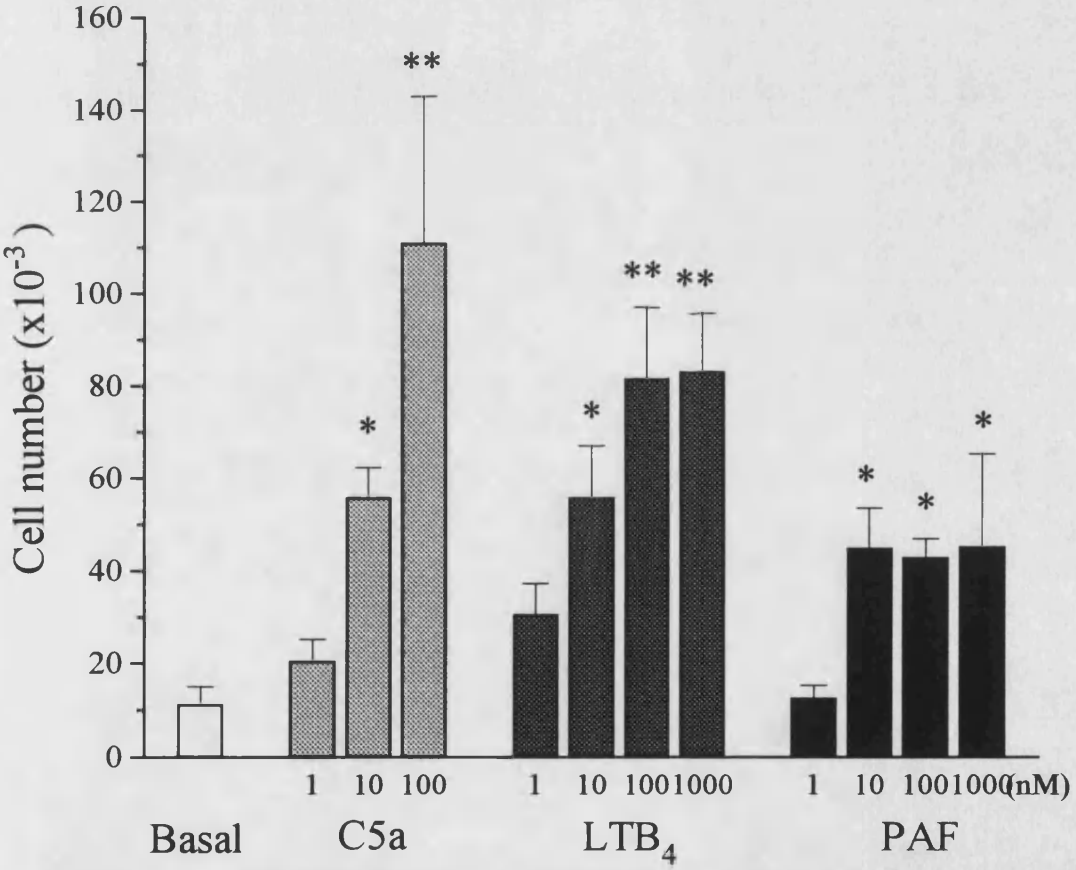


Figure 4.3 Adhesion of guinea pig eosinophils stimulated with C5a (1-100nM)(▒), LTB_4 (1nM-1μM)(▩) or PAF (1nM-1μM)(■) at t=90min. Bars indicate mean±sem total number of cells adhered to the plate after the H_2O_2 assay for n=3-7 different eosinophil preparations. Significantly increased adhesion compared with basal cellular adhesion: *, $p<0.05$; **, $p<0.01$.

When guinea pig eosinophils were stimulated with C5a, LTB_4 and PAF all

agonists were able to cause significant adherence of eosinophils at both 10nM and 100nM (figure 4.3). At 10nM all agonists had approximately the same effect on adhesion, whereas they had differing effects on H_2O_2 production (see figure 3.4). As with H_2O_2 release LPS, fMLP and IL-8 had no effect on guinea pig adhesion.

4.2.2 Effect of extracellular cationic conditions on adhesion

Similar to the inhibition of H_2O_2 production, the absence of extracellular calcium and magnesium caused inhibition of guinea pig adhesion to laminin-coated microtitre plates. This was significant for C5a (100nM) and PMA (30nM) stimulated cells, however significance was not acheived for either LTB_4 or PAF treated cells due to poor adhesion in positive controls (figure 4.4). In buffer containing calcium only (1mM), the effect of adhesion was quite variable as shown by the large error bars, but as a general rule, the adhesion of eosinophils was only effected when both calcium and magnesium were absent.

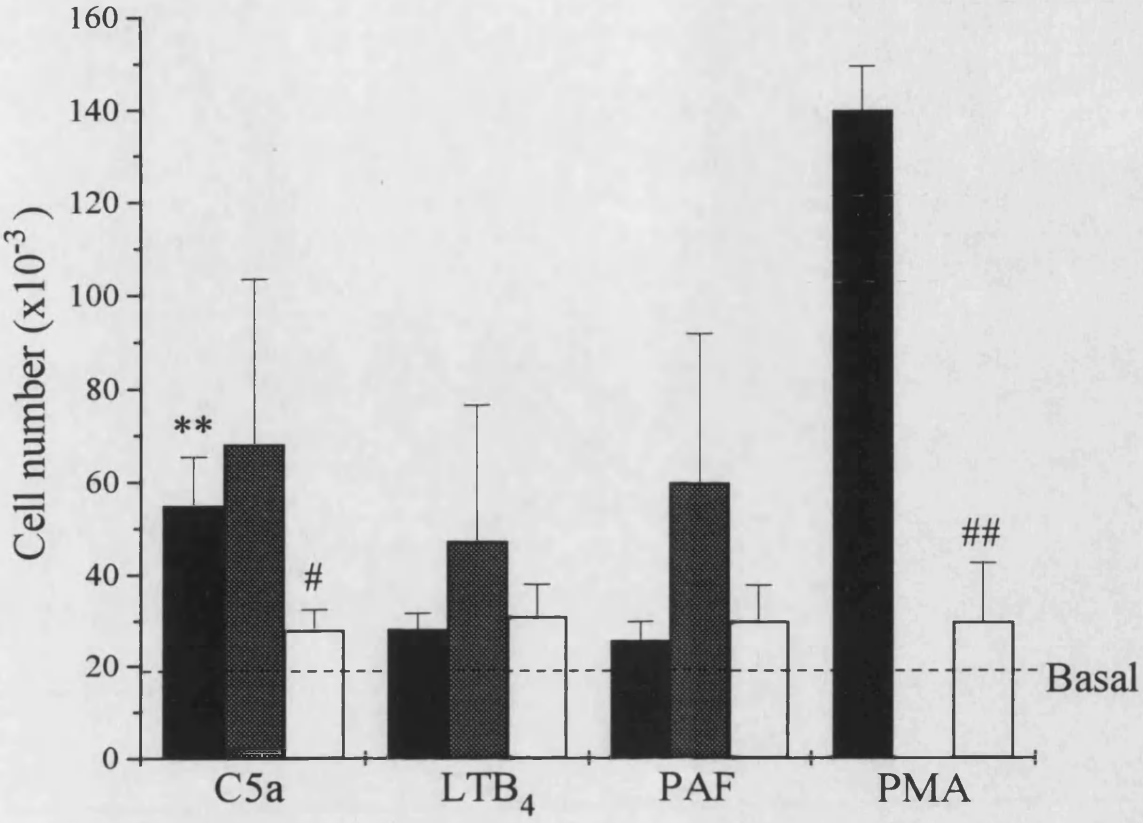


Figure 4.4 Adhesion of guinea pig eosinophils stimulated with C5a (100nM), LTB_4 (100nM), PAF (100nM) and PMA (30nM) to laminin-coated microtitre plates in the presence of 1mM Ca^{2+}/Mg^{2+} (■), 1mM EGTA (▩) or 1mM EDTA (□) at t=90min. Bars indicate mean±sem total number of eosinophils adhered after H_2O_2 assay for n=3-9 different eosinophil preparations. Significantly increased adhered cells compared with basal cellular adhesion: **, $p<0.01$. Significantly reduced adhesion compared with 1mM Ca^{2+}/Mg^{2+} containing cells: #, $p<0.05$; ##, $p<0.01$.

4.2.3 Effect of extracellular nickel on adhesion

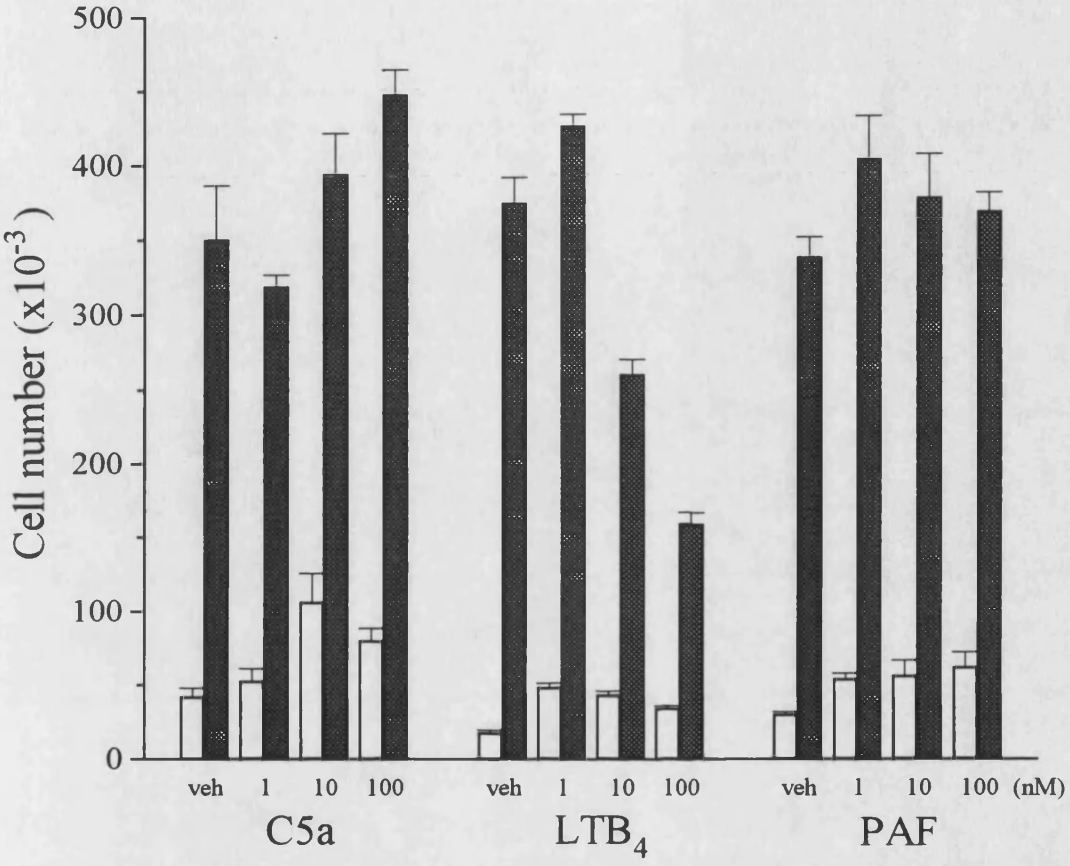


Figure 4.5 Adhesion of guinea pig eosinophils stimulated with C5a (1-100nM), LTB_4 (1-100nM) or PAF (1-100nM) to laminin-coated microtitre plates in the presence of Ca^{2+}/Mg^{2+} (1mM)(□) or $NiCl_2$ (1mM)(▩) at t=90 min. Bars indicate mean±sem total number of cells adhered after H_2O_2 assay for triplicate wells. Representative of 2 experiments.

Though extracellular nickel (1mM) had no effect on H_2O_2 production from adherent guinea pig eosinophils by any agonist tested, nickel had a marked effect on adhesion. The number of adhered cells was increased by more than 300% for vehicle, C5a (1-100nM) and PAF (1-100nM) treated cells. Interestingly on both occasions tested, as the dose of LTB_4 increased, the effect of increased adhesion caused by the presence of extracellular nickel was reduced (figure 4.5).

4.2.4 Effect of SK&F 96365 on adhesion

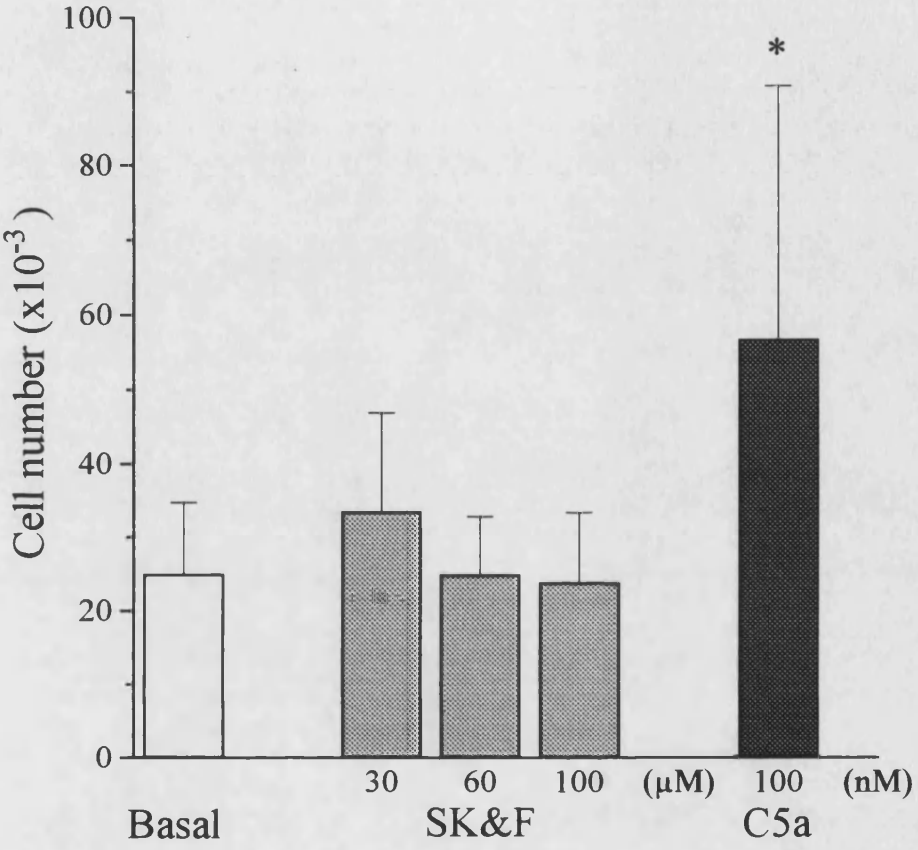


Figure 4.6 Adhesion of guinea pig eosinophils stimulated with SK&F 96365 (30-100μM)(▒) or C5a (100nM)(▩) to laminin-coated microtitre plate at t=90min. Bars indicate mean±sem total number of cells adhered after H_2O_2 assay for n=3 different eosinophil preparations. Significantly increased adhered cells compared with basal cellular adhesion: *, $p<0.05$.

The ROCC blocker SK&F 96365 was also tested for its effect on adhesion. At all concentrations tested (30-100μM), SK&F 96365 had no significant effect on adhesion (figure 4.6). This is very different to the result SK&F 96365 has on H_2O_2 production from guinea pig eosinophils (figure 3.9). When SK&F 96365 was tested in conjunction with C5a (100nM) and PAF (100nM) the adhesion was markedly reduced as the concentration of SK&F 96365 was increased (figure 4.7).

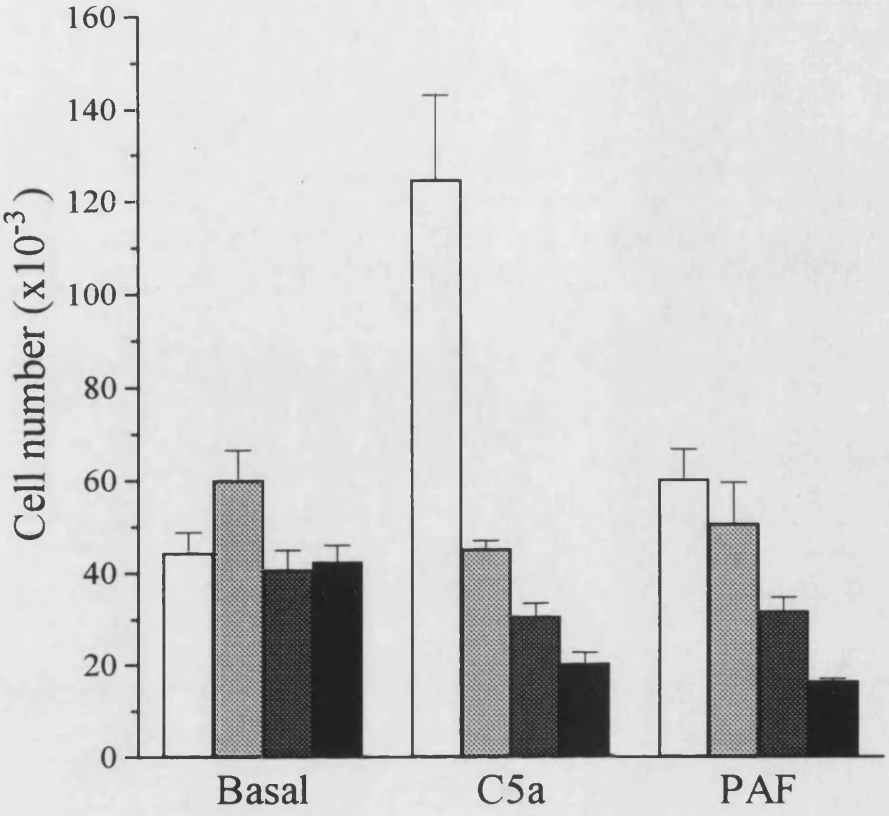


Figure 4.7 Adhesion of guinea pig eosinophils either unstimulated or stimulated with C5a (100nM) or PAF (100nM) in conjuction with vehicle (□), 30μM (▒), 60μM (▓) or 100μM (■) of SK&F 96365 to laminin-coated microtitre plates at t=90min. Bars indicate mean±sem total number of cells adhered after H_2O_2 assay for triplicate wells. Representative of 2 experiments.

4.2.5 Effect of the specific PKC inhibitor, Ro 31-8220/002, on adhesion

Previous experiments (figure 4.3) indicated a dissociation of mechanisms of action between H_2O_2 production and cellular adhesion existed. When guinea pig eosinophils were incubated with the PKC inhibitor Ro 31-8220/002 for 20 min at 37°C, an effect on adhesion was detected after the H_2O_2 assay.

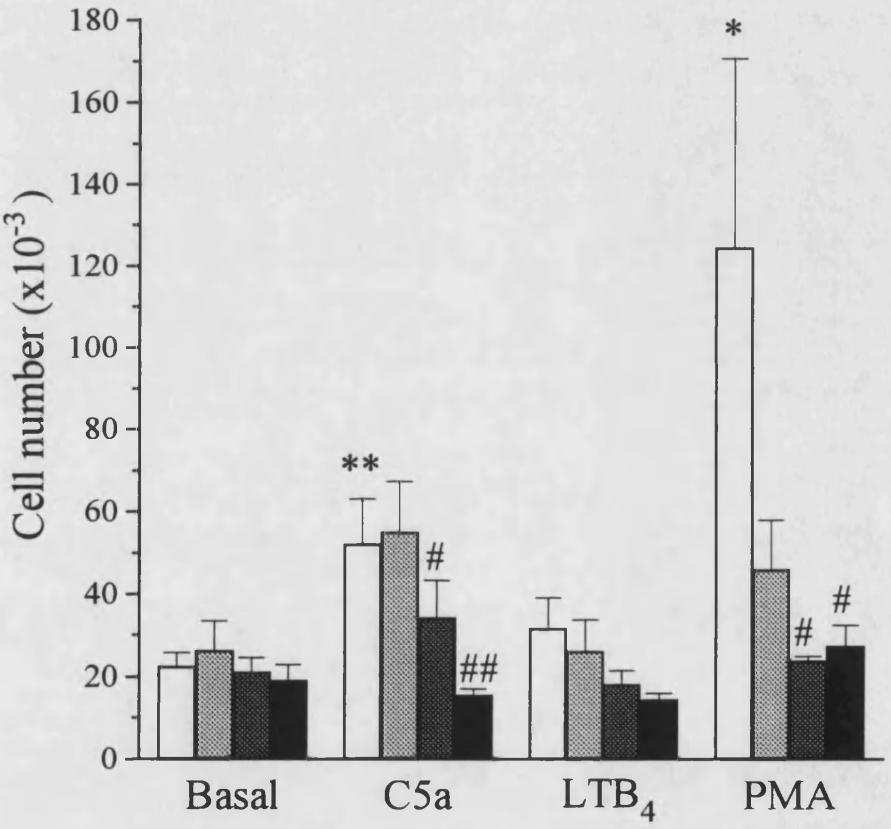


Figure 4.8 Adhesion of guinea pig eosinophils pretreated with vehicle (□), 300nM (▒), 1μM (▩), 3μM (■) of the PKC selective inhibitor, Ro 31-8220/002 (for 20min at 37°C before stimulation), with C5a (30nM), LTB_4 (30nM) or PMA (300pM) to laminin-coated microtitre plates at t=90min. Bars indicate mean±sem total number of cells adhered after H_2O_2 assay for n=4-7 different eosinophil preparations. Significantly increased adhesion compared with basal cellular adhesion: *, $p<0.05$; **, $p<0.01$. Significantly reduced adhesion compared with vehicle treated cells: #, $p<0.05$; ##, $p<0.01$.

The adhesion of eosinophils was inhibited dose-dependently in C5a (30nM), LTB_4 (30nM) and PMA (300pM) stimulated cells. This was only significant in C5a and PMA stimulated cells as LTB_4 did not significantly increase eosinophil adhesion above basal levels in vehicle treated cells (figure 4.8).

4.2.6 Effect of the tyrosine kinase inhibitors, herbimycin A and genistein on adhesion

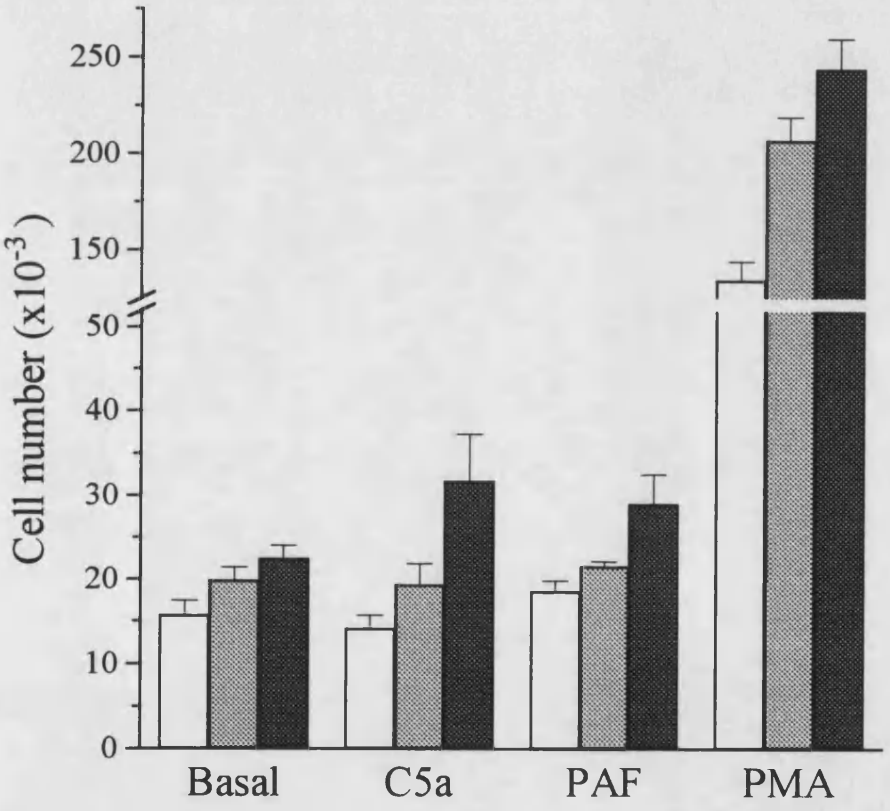


Figure 4.9 Adhesion of guinea pig eosinophils pretreated with vehicle (□) or the tyrosine kinase inhibitors herbimycin A (1μM)(▒) or genistein (100μM)(■) (for 2 hours at 37°C) before stimulation with C5a (100nM), PAF (100nM) or PMA (3nM) to laminin-coated microtitre plates at t=90min. Bars indicate mean±sem total number of cells adhered after H_2O_2 assay for triplicate wells. Representative of 2 experiments.

The effect of a 2½ hour preincubation of eosinophils with tyrosine kinase

inhibitors on cellular adhesion was also measured after H_2O_2 production. Both herbimycin A (100μM) and genistein (100μM) enhanced the adhesion of eosinophils stimulated with C5a (100nM), PAF (100nM) or PMA (3nM) to laminin-coated microtitre plates above control levels (figure 4.9). These control levels were measurably lower than those detected in figure 4.3. This was probably due to the long preincubation times with the tyrosine kinase inhibitors, but the eosinophils were still >99% viable after the H_2O_2 assay.

4.2.7 Effect of wortmannin on adhesion

The effect of the specific PI 3-kinase inhibitor, wortmannin was also tested on the adhesion of eosinophils. Figure 4.10 shows that in C5a (100nM), PAF (100nM) or PMA (1nM) stimulated cells, the effect of adhesion was always reduced by wortmannin (3-30nM), however this was not a dose-related effect.

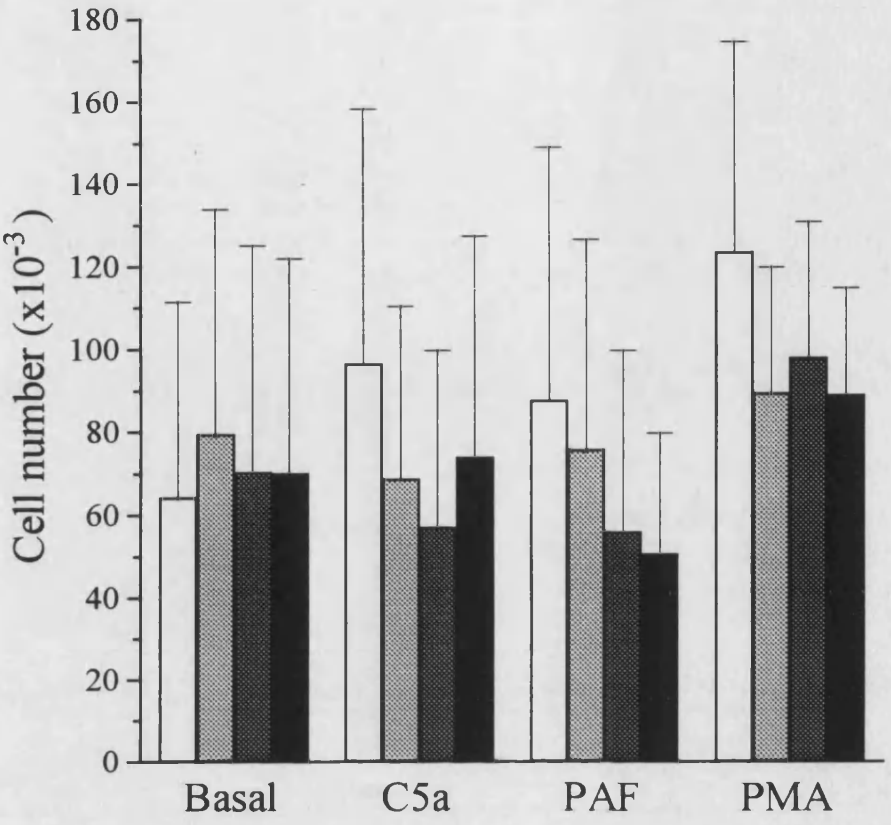


Figure 4.10 Adhesion of guinea pig eosinophils pretreated with vehicle (□) 3nM (▒), 10nM (▓) or 30nM (■) of the PI 3-kinase inhibitor wortmannin (for 10min at 37°C), before stimulation with C5a (100nM), PAF (100nM) or PMA (1nM) to laminin-coated microtitre plates at t=60min. Bars indicate mean±sem total number of cells adhered after H_2O_2 assay for n=3 different eosinophil preparations.

4.2.8 Adhesion of BAL eosinophils stimulated with different agonists

In addition to guinea pig eosinophils obtained by peritoneal lavage, BAL eosinophils were also harvested from control and OA-challenged guinea pigs, to determine if there were any differences between the different ways the cells were elicited. The levels of adhesion of BAL eosinophils acquired from OA-challenged animals were always greater than the levels of adhesion from BAL eosinophils obtained from non-/sham-challenged animals in C5a (30-100nM) and PAF (30-100nM)

stimulated cells as shown in figure 4.11. Experiments with BAL eosinophils from non-/sham-challenged animals and PMA (3nM) were not done.

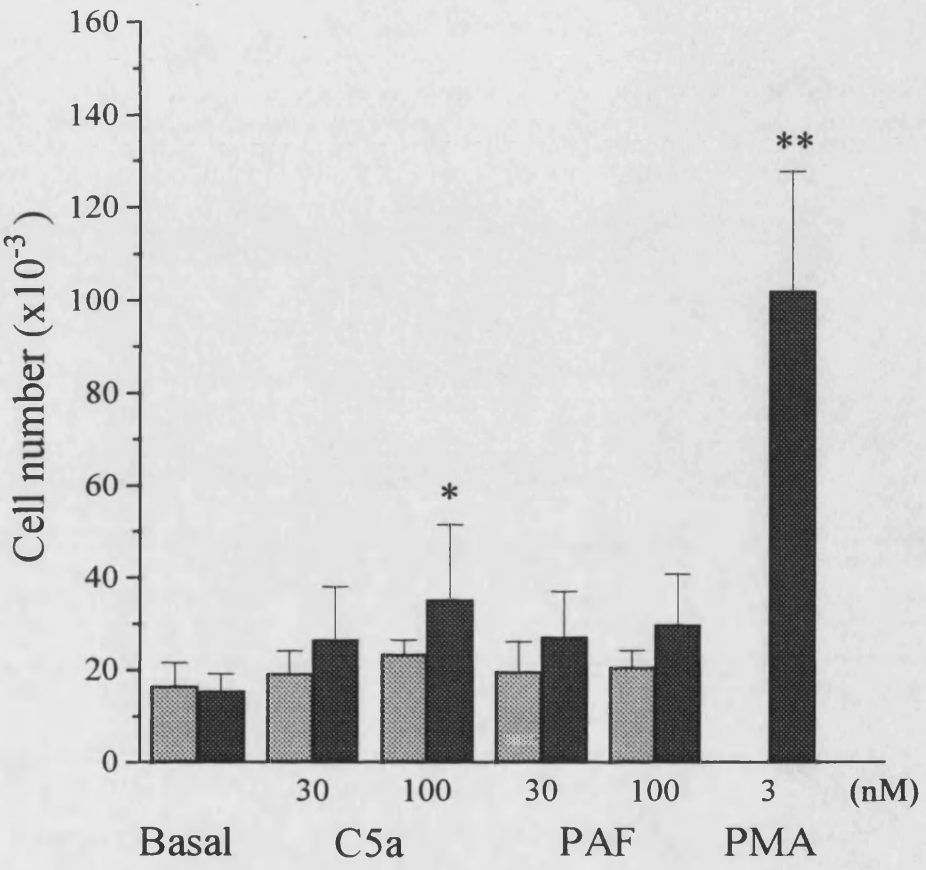


Figure 4.11 Adhesion of guinea pig BAL eosinophils of non-/sham-challenged animals (▒) or OA-challenged (▩) stimulated with C5a (30-100nM), PAF (30-100nM) or PMA (3nM) to laminin-coated microtitre plates at t=90min. Bars indicate mean±sem total number of cells adhered after H_2O_2 asay for n=3-5 different eosinophil preparations. Significantly increased adhesion compared with relative-basal control cellular adhesion: *, $p<0.05$; **, $p<0.01$.

4.3 Human eosinophils

4.3.1 Effect of added agonists, including the C-C chemokines and cytokines, on adhesion

C5a (100nM), IL-5 (40nM) and PMA (1nM) could significantly increase the adhesion of human eosinophils to laminin-coated microtitre plates above basal levels. MCP-3 (100nM) and RANTES (100nM) always stimulated a greater level of adhesion compared to basal levels, but did not reach significance (figure 4.12).

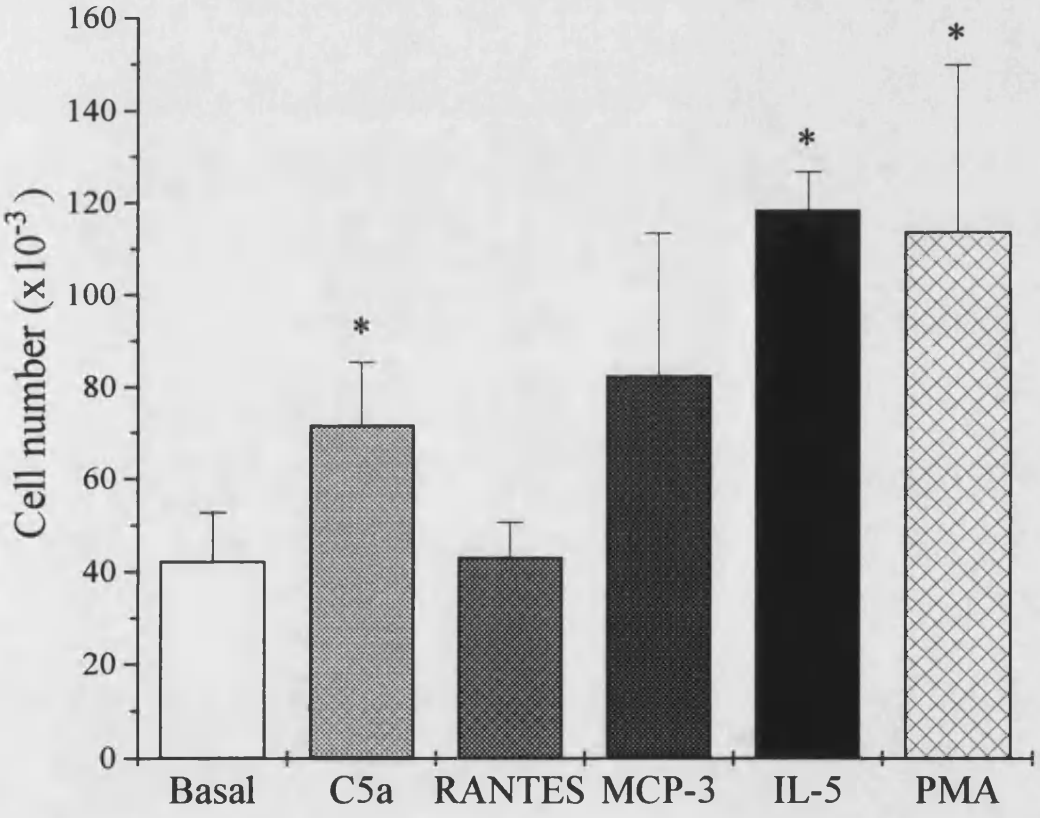


Figure 4.12 Adhesion of human eosinophils stimulated with C5a (100nM)(▒), RANTES (100nM)(▨), MCP-3 (100nM)(▩), IL-5 (40nM)(■) and PMA (1nM)(⊠) to laminin-coated microtitre plates at t=60min. Bars indicate mean±sem total number of cells adhered after H_2O_2 assay for n=3-6 different eosinophil preparations. Signifcantly increased adhesion compared with basal cellular adhesion: *, $p<0.05$.

Table 4.2 Effect of MIP-1α/β and MCP-3 on adhesion of human eosinophils at t=60min. Data indicates mean±sem total number of cells adhered after H_2O_2 assay from triplicate wells. Representative of 2 experiments.

Agonist	Cells adhered ($x10^{-4}$) at t=60min
Basal	3.21±0.49
MIP-1α (100nM)	2.90±0.03
MIP-1β (100nM)	3.45±0.50
MCP-1 (100nM)	3.49±0.67
C5a (100nM)	7.62±0.40

The C-C chemokines MIP-1α (100nM), MIP-1β (100nM) and MCP-1 (100nM) had no effect on adhesion (table 4.2).

4.3.2 Effect of Ro 31-8220/002 on adhesion

The effect of the selective PKC inhibitor, Ro 31-8220/002, on human eosinophil adhesion was also determined. Ro 31-8220/002 (0.3-3μM) dose-dependently inhibited the adhesion of eosinophils stimulated by PMA (1nM), MCP-3 (100nM) and IL-5 (40nM). Interestingly, in unstimulated and C5a (100nM) stimulated eosinophils, Ro 31-8220/002 (0.3-3μM) appeared to enhance the adhesion of human eosinophils (figure 4.13). However when the basal levels of adhesion was subtracted from the levels of C5a stimulated eosinophil adhesion, there was no net increase in adhesion.

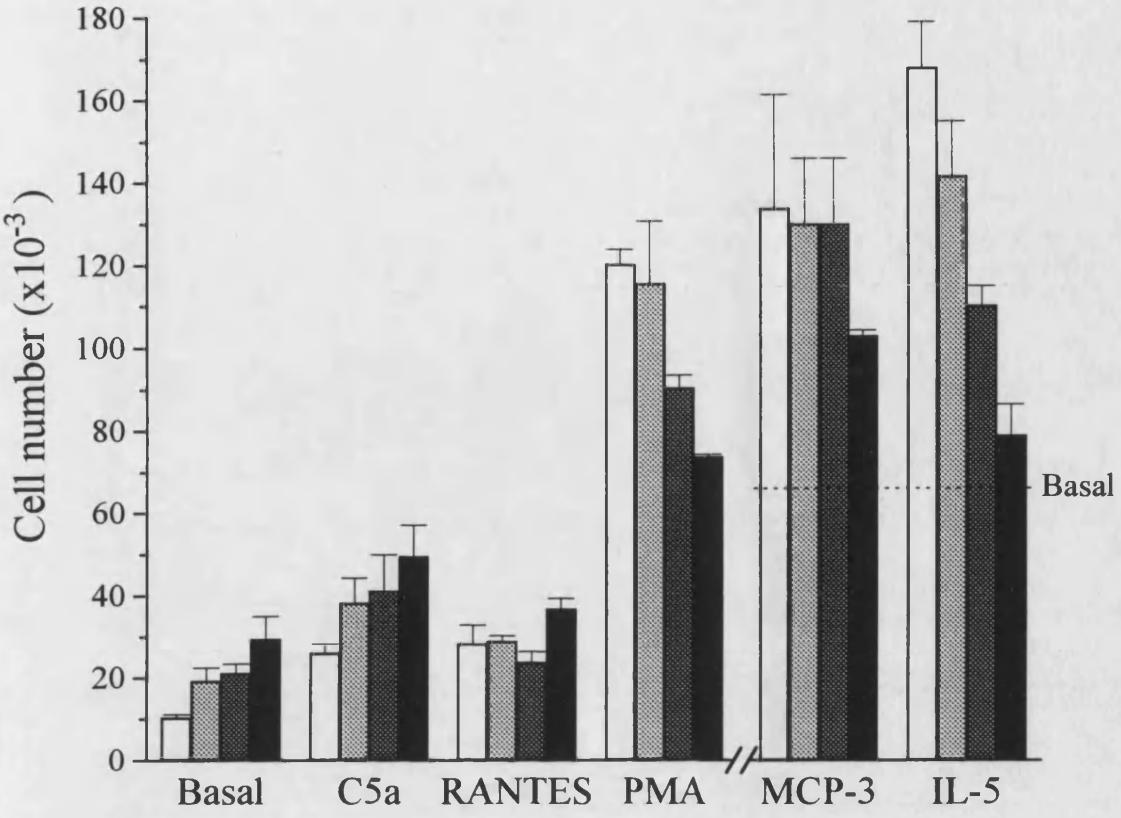


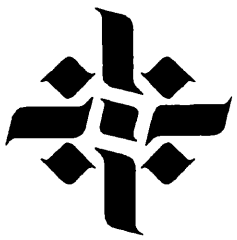
Figure 4.13 Adhesion of human eosinophils pretreated with vehicle (□), 300nM (▒), 1μM (▩) or 3μM (■) of the selective PKC inhibitor Ro 31-8220/002 (for 20min at 37°C), before stimulation with C5a (100nM), RANTES (100nM), PMA (1nM), MCP-3 (100nM) or IL-5 (40nM) to laminin-coated microtitre plates at t=60min. Bars indicate mean±sem total number of cells adhered after H_2O_2 assay for triplicate wells. Representative of 2 experiments. Experiment with MCP-3 and IL-5 was conducted on a different eosinophil preparation that had a higher basal adhesion as indicated by the dotted line.

4.4 Summary of adhesion experiments

- Guinea pig and human eosinophils have enhanced adhesion when stimulated with particular soluble stimuli.
- Guinea pig eosinophils have a significantly increased adhesion when stimulated by C5a, LTB_4, PAF and PMA.
- Human eosinophils have a significantly increased adhesion when stimulated by

C5a, MCP-3, IL-5 and PMA. RANTES also augments adhesion.

- Guinea pig eosinophil adhesion is dependent on calcium and magnesium.
- Guinea pig eosinophil adhesion is enhanced when extracellular nickel is present.
- The ROCC blocker SK&F 96365 does not effect guinea pig eosinophil adhesion, but when used in conjunction with the added agonists C5a and PAF, SK&F 96365 inhibited the adhesion of eosinophils.
- Adhesion of guinea pig eosinophils was enhanced by the tyrosine kinase inhibitors herbimycin A and genistein.
- Guinea pig eosinophil adhesion could be inhibited by nanomolar concentrations of the specific PI 3-kinase inhibitor, wortmannin.
- Both guinea pig and human eosinophil adhesion could be dose-dependently inhibited by micromolar concentrations of the selective PKC inhibitor, Ro 31-8220/002.



5 Eosinophil Intracellular Free Calcium Elevation

5.1 Guinea pig eosinophil

5.1.1 Effect of the added agonists C5a, LTB_4 and PAF

The guinea pig eosinohil prepartions used had a basal intracellular free calcium concentration ($[Ca^{2+}]_i$) of 59±9nM (n=12). Upon stimulation with C5a, LTB_4 and PAF all agonists produced characteristic responses (figure 5.1). Elevations in $[Ca^{2+}]_i$ stimulated with PAF were less rapid than those shown by C5a and LTB_4. Furthermore it was demonstrated that LTB_4 elicited a biphasic response at 1nM and above. All three agonists produced dose related increases of $[Ca^{2+}]_i$ above basal levels. The maximal elevation in response to each agonist was similar though PAF and C5a had maximal effects at 100nM whereas maximal LTB_4 responses were observed between 1nM and 10nM. This was emphasised when analyzing the data over 5-6 independent experiments (figure 5.2). Each agonist at 10nM and 100nM induced similar significant elevations in $[Ca^{2+}]_i$, whereas LTB_4 was the only agonist to stimulate a significant rise of $[Ca^{2+}]_i$ at 1nM. Again as in the H_2O_2 experiments fMLP, LPS and IL-8 had little effect on the cells (table 5.1). In addition, other work in the laboratories has shown that human chemokines are also unable to induce $[Ca^{2+}]_i$ elevation in guinea pig eosinophils (data not shown).

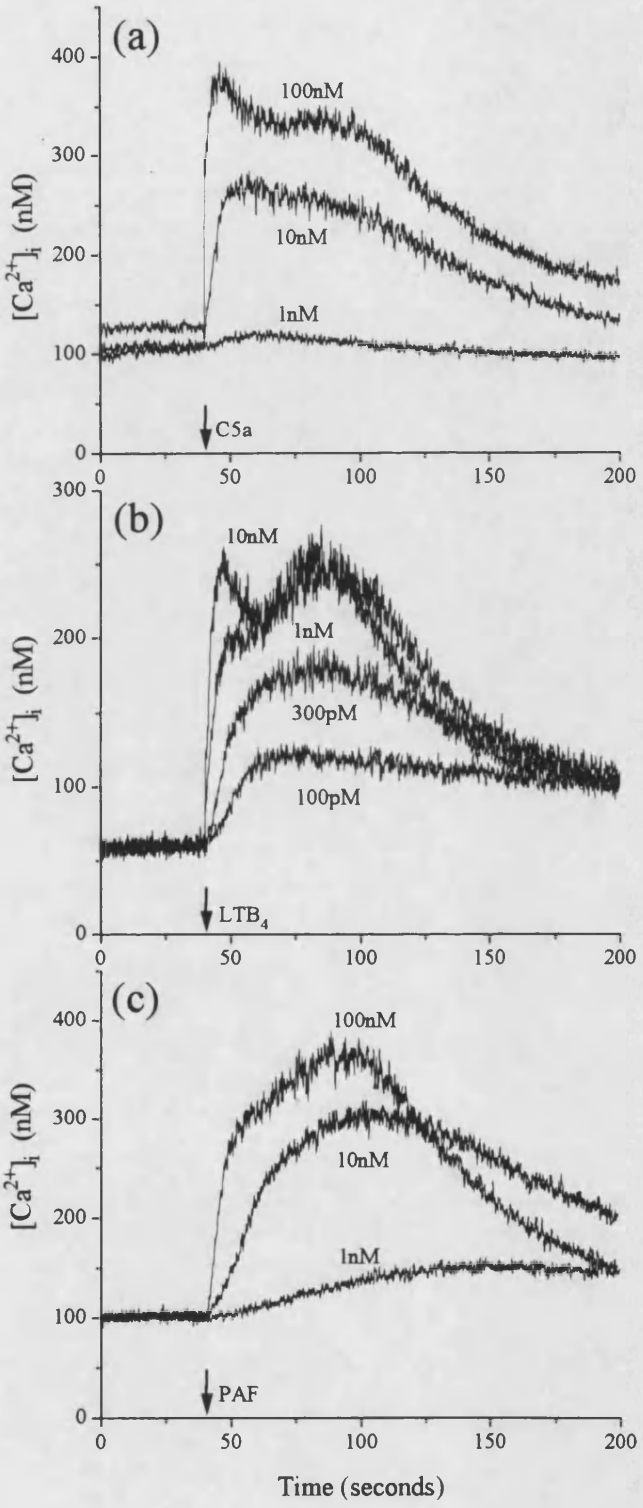


Figure 5.1 Time courses of $[Ca^{2+}]_i$ elevation in fura-2-loaded guinea pig eosinophils ($2x10^6$) stimulated with (a) C5a (1-100nM), (b) LTB_4 (0.1-10nM) or (c) PAF (1-100nM). Traces are representative of 5-6 experiments.

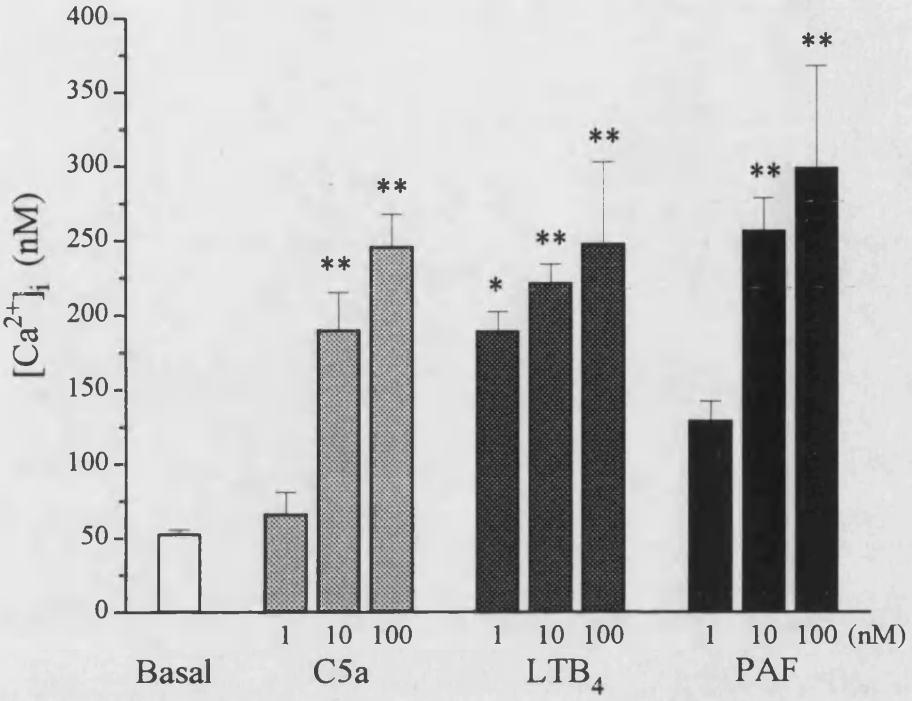


Figure 5.2 $[Ca^{2+}]_i$ levels in fura-2-loaded guinea pig eosinophils either unstimulated (□) or stimulated with C5a (▒), LTB_4 (▩) or PAF (■). Bars indicate mean±sem maximal $[Ca^{2+}]_i/2x10^6$cells for n=5-6 different eosinophil preparations. Significantly increased $[Ca^{2+}]_i$ elevation compared with basal $[Ca^{2+}]_i$ levels: *, $p<0.05$; **, $p<0.01$.

Table 5.1 Effects of IL-8, fMLP and LPS on $[Ca^{2+}]_i$ in guinea pig eosinophils. Data indicates mean±sem for maximal $[Ca^{2+}]_i/2x10^6$ cells for n=3-5 different eosinophil preparations.

Agonist	$[Ca^{2+}]_i$ (nM)
Basal	59.0±9.0
IL-8 (10nM)	72.7±2.3
fMLP (100nM)	79.6±4.3
LPS (10μg/ml)	63.9±0.8
C5a (100nM)	243.5±25.8

5.1.2 Effect of extracellular ionic conditions

The effect of varying the extracellular ions on free calcium elevation was the same for each agonist tested *viz.* C5a, LTB_4 and PAF (10nM). In the presence of calcium (1mM) only, there seemed to be little change in agonist-induced elevation of free calcium. In the presence of magnesium (1mM) only, in nominal calcium and magnesium (50-100μM) or in the presence of the calcium chelator EGTA (1mM), the maximal $[Ca^{2+}]_i$ elevated was markedly reduced by 80-90% (figures 5.3 and 5.4). This is in contrast to H_2O_2 experiments where extracellular magnesium was found to be an important factor (figure 3.5). From figure 5.4 it can be seen that the basal $[Ca^{2+}]_i$ was reduced in cells that had nominal or no extracellular calcium suggesting some calcium store depletion.

Figure 5.3 (see next page) Time courses of $[Ca^{2+}]_i$ elevation in fura-2-loaded guinea pig eosinophils ($2x10^6$) stimulated at 40 seconds with (a) C5a (10nM), (b) LTB_4 (10nM) or (c) PAF (10nM) in the presence of (i) Ca^{2+}/Mg^{2+} (1mM), (ii) Ca^{2+} (1mM) only, (iii) Mg^{2+} (1mM) only, (iv) nominal Ca^{2+}/Mg^{2+} (~50-100μM) or (v) EGTA (1mM). Traces are representative of 3 experiments.

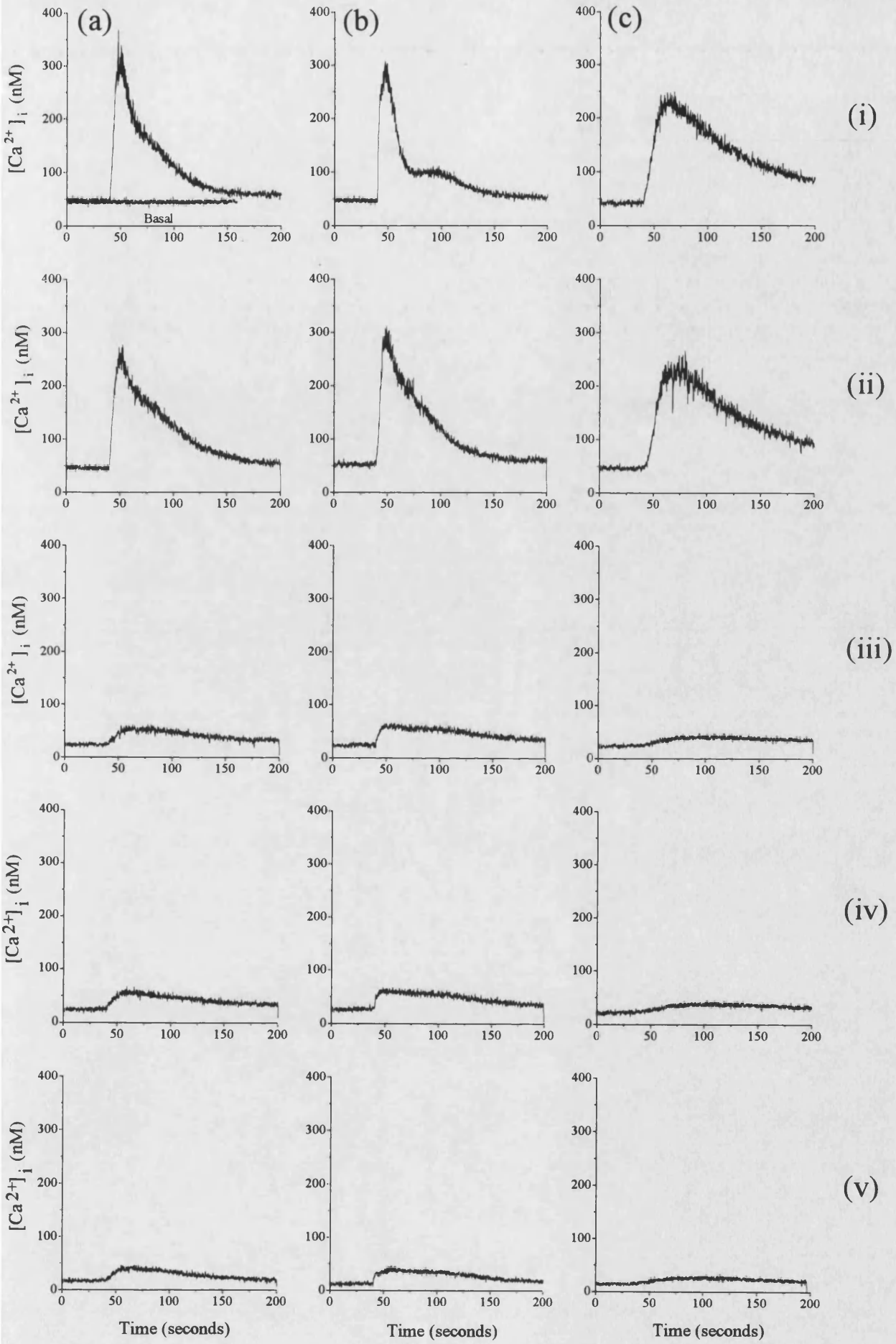

(a)
(b)
(c)
(i)
(ii)
(iii)
(iv)
(v)
[Ca2+]i (nM)
Basal
Time (seconds)
Time (seconds)
Time (seconds)

Figure 5.3

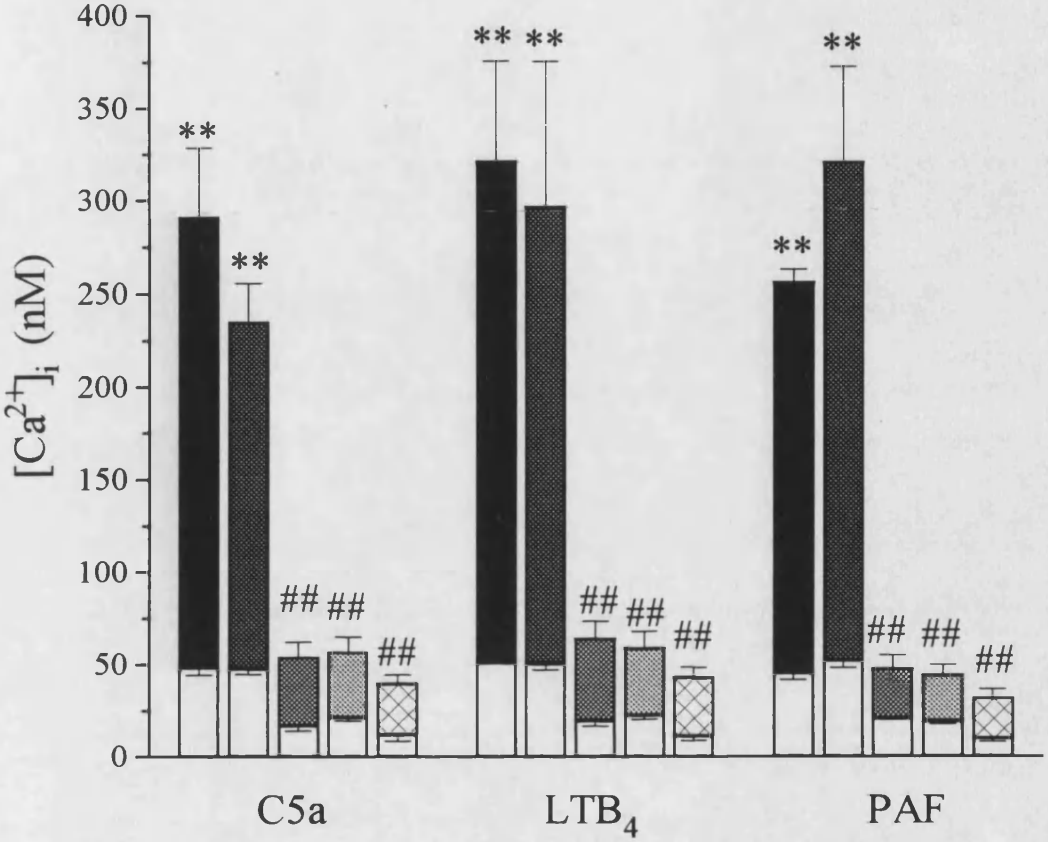


Figure 5.4 $[Ca^{2+}]_i$ levels in fura-2-loaded guinea pig eosinophils stimulated with C5a, LTB_4 or PAF (10nM) in the presence of Ca^{2+}/Mg^{2+} (1mM)(■), Ca^{2+} (1mM) only (▩), Mg^{2+} (1mM) only (▨), nominal Ca^{2+}/Mg^{2+} (~50-100μM)(▒) or EGTA (1mM)(□). Basal $[Ca^{2+}]_i$ levels (□). Bars indicate mean±sem maximal $[Ca^{2+}]_i/2x10^6$cells for n=3 different eosinophil preparations. Significantly increased $[Ca^{2+}]_i$ elevation compared with basal $[Ca^{2+}]_i$ levels: **, $p<0.01$. Significantly reduced $[Ca^{2+}]_i$ elevation compared with Ca^{2+}/Mg^{2+} (1mM) containing cells: ##, $p<0.01$.

5.1.3 Effect of nickel on $[Ca^{2+}]_i$

The previous experiments suggested that for $[Ca^{2+}]_i$ elevation, extracellular calcium was important. Thus nickel, which blocks membrane calcium channels and subsequent influx into the cell, was used to investigate the role of extracellular calcium. Nickel which does not interfere with fura-2 fluoresence, also has the advantage that

extracellular Ca^{2+} (1mM) can be present, thus minimising the effects of calcium store depletion. Figure 5.5 demonstrates that nickel (1mM) reduced the elevation of $[Ca^{2+}]_i$ in C5a (10nM), LTB_4 (10nM) and PAF (10nM) stimulated eosinophils by a small amount. In most cases it removed the second peak in the LTB_4 biphasic response suggesting that this increase was due to calcium influx. Figure 5.6 shows the pooled data and indicates a consistent reduction (though not significant) of agonist-induced $[Ca^{2+}]_i$ elevation in guinea pig eosinophils. This inhibition was greater than the reduction which occurred on basal $[Ca^{2+}]_i$ levels when nickel was added.

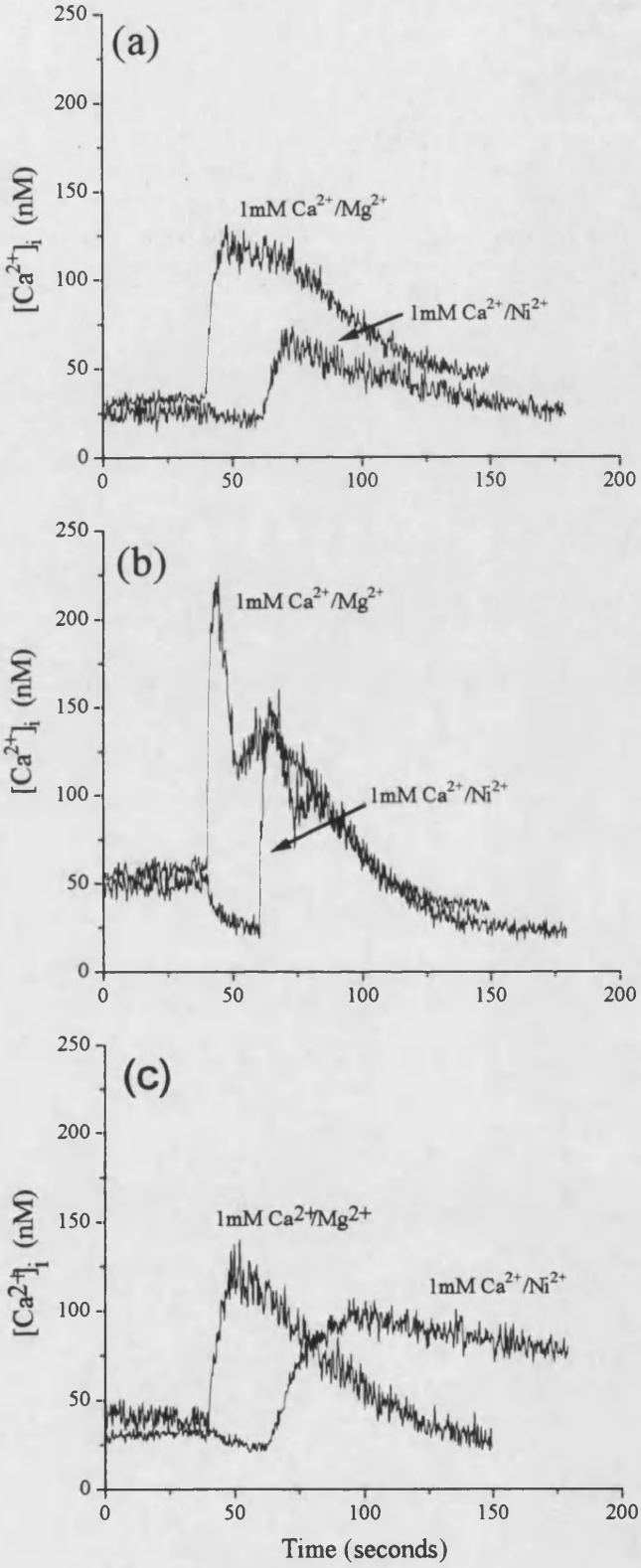


Figure 5.5 Time courses of $[Ca^{2+}]_i$ elevation in fura-2 loaded guinea pig eosinophils ($2x10^6$) stimulated with (a) C5a (10nM), (b) LTB_4 (10nM) or (c) PAF (10nM) in the presence of extracellular Ca^{2+}/Mg^{2+} (1mM) or Ca^{2+}/Ni^{2+} (1mM). Agonists added at 40 seconds for Ca^{2+}/Mg^{2+} experiments and at 60 seconds (20 seconds post-nickel addition) for Ca^{2+}/Ni^{2+} experiments. Traces are representative of 3 experiments.

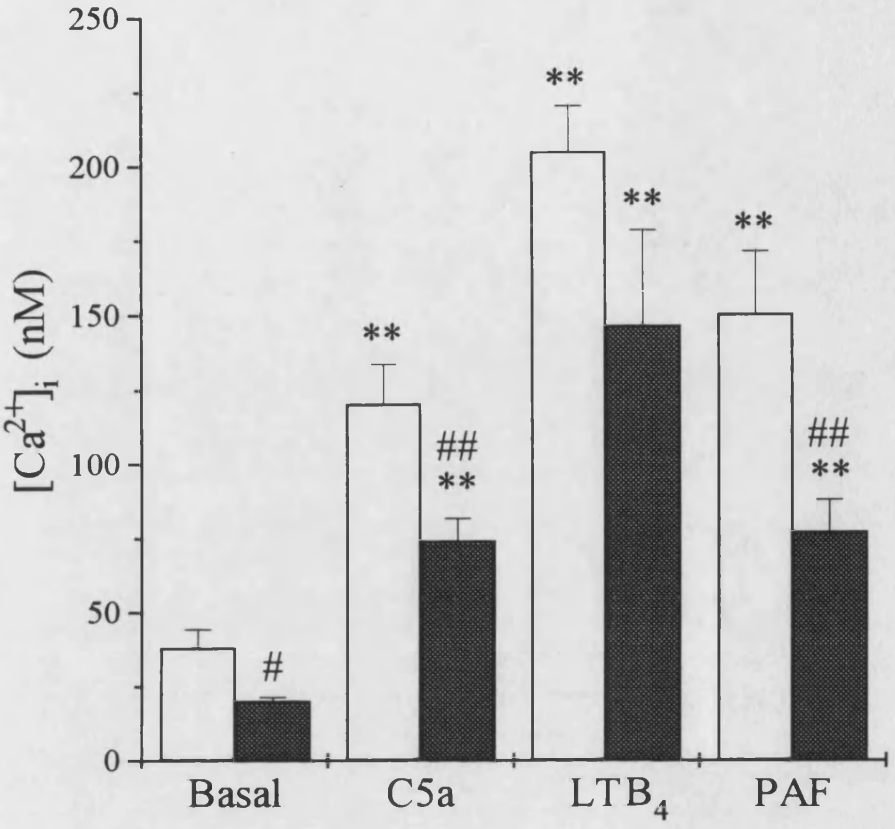


Figure 5.6 $[Ca^{2+}]_i$ levels in fura-2 loaded guinea pig eosinophils either unstimulated or stimulated with C5a, LTB_4 or PAF (10nM) in the presence of Ca^{2+}/Mg^{2+} (1mM)(□) or Ca^{2+}/Ni^{2+} (1mM)(▩). Bars indicate mean±sem maximal $[Ca^{2+}]_i/2x10^6$cells for n=3 different eosinophil preparations. Significantly increased $[Ca^{2+}]_i$ elevation compared to respective basal $[Ca^{2+}]_i$ levels: **, $p<0.01$. Significantly reduced $[Ca^{2+}]_i$ elevation compared with cells in the presence of Ca^{2+}/Mg^{2+}: #, $p<0.05$; ##, $p<0.01$.

5.1.4 Effect of manganese on $[Ca^{2+}]_i$

To investigate further the role calcium influx in guinea pig eosinophils, extracellular manganese was used. Manganese can be used as a surrogate ion for calcium in influx experiments and it has the additional property of having a greater affinity for fura-2 than calcium. Experiments were performed at fura-2's isobestic point (360nm). Figure 5.7 demonstrates how useful manganese is in detecting influx by comparing the effect of ionomycin (10μM) to vehicle-treated cells.

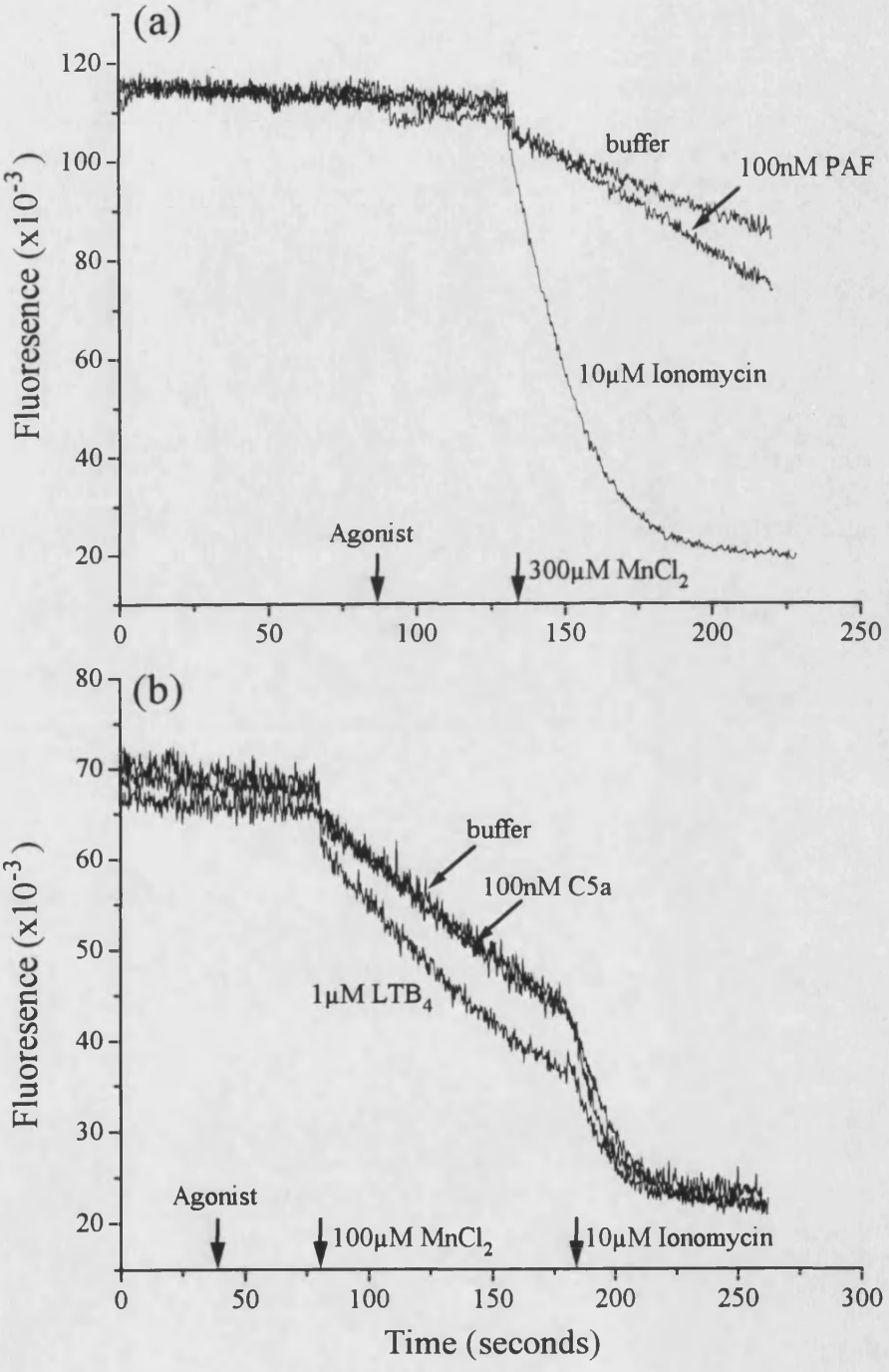


Figure 5.7 Time courses of fluoresence in fura-2-loaded guinea pig eosinophils ($2x10^6$) either unstimulated or stimulated with PAF (100nM), C5a (100nM), LTB_4 (1μM) or ionomycin (10μM) in the presence of Ca^{2+} (50μM)/Mg^{2+} (1mM) with either (a) 300μM $MnCl_2$ or (b) 100μM $MnCl_2$ added (Ex=360nm and Em=510nm). Traces are representative of 2-3 experiments.

In contrast, PAF (100nM), C5a (100nM) and LTB_4 (1μM) cause very small amounts of manganese influx, if any, when comparing vehicle-treated cells. This implies calcium influx is a minor component of the signalling system and the suppression seen in section 5.1.2 was likely due to depletion of intracellular calcium stores.

5.1.5 Effect of SK&F 96365 on $[Ca^{2+}]_i$

The specific ROCC blocker SK&F 96365 (10-60μM) was tested for its effect on $[Ca^{2+}]_i$. Figure 5.8 demonstrates that SK&F 96365 (10-60μM) caused a concentration-dependent increase in $[Ca^{2+}]_i$ in guinea pig eosinophils. This was followed by a concentration-dependent inhibition of $[Ca^{2+}]_i$ elevation in response to C5a (10nM) and PAF (10nM). This effect was also observed for the enantiomers of SK&F 96365 and these appeared to be as effective as each other. Figure 5.9 shows that the addition of PAF (10nM) before SK&F 96365 (60μM) had no effect on elevation of $[Ca^{2+}]_i$. Figure 5.10 shows the pooled data of the 'agonist' effect of SK&F 96365 and its enantiomers on $[Ca^{2+}]_i$ and figure 5.11 shows the pooled data of the inhibitory effects SK&F 96365 and its enantiomers on $[Ca^{2+}]_i$ in guinea pig eosinophils.

Figure 5.8 (see next page) Time courses of $[Ca^{2+}]_i$ elevation in fura-2-loaded guinea pig eosinophils ($2x10^6$) either stimulated with (i) SK&F 96365 (10-60μM), (ii) (-) SK&F 96365 (10-60μM) or (iii) (+)SK&F 96365 (10-60μM) then either (a) C5a (10nM) or (b) PAF (10nM). In panel (i), control experiments with no SK&F 96365 are shifted by 150nM for clarity. Traces are representative of 3-6 experiments.

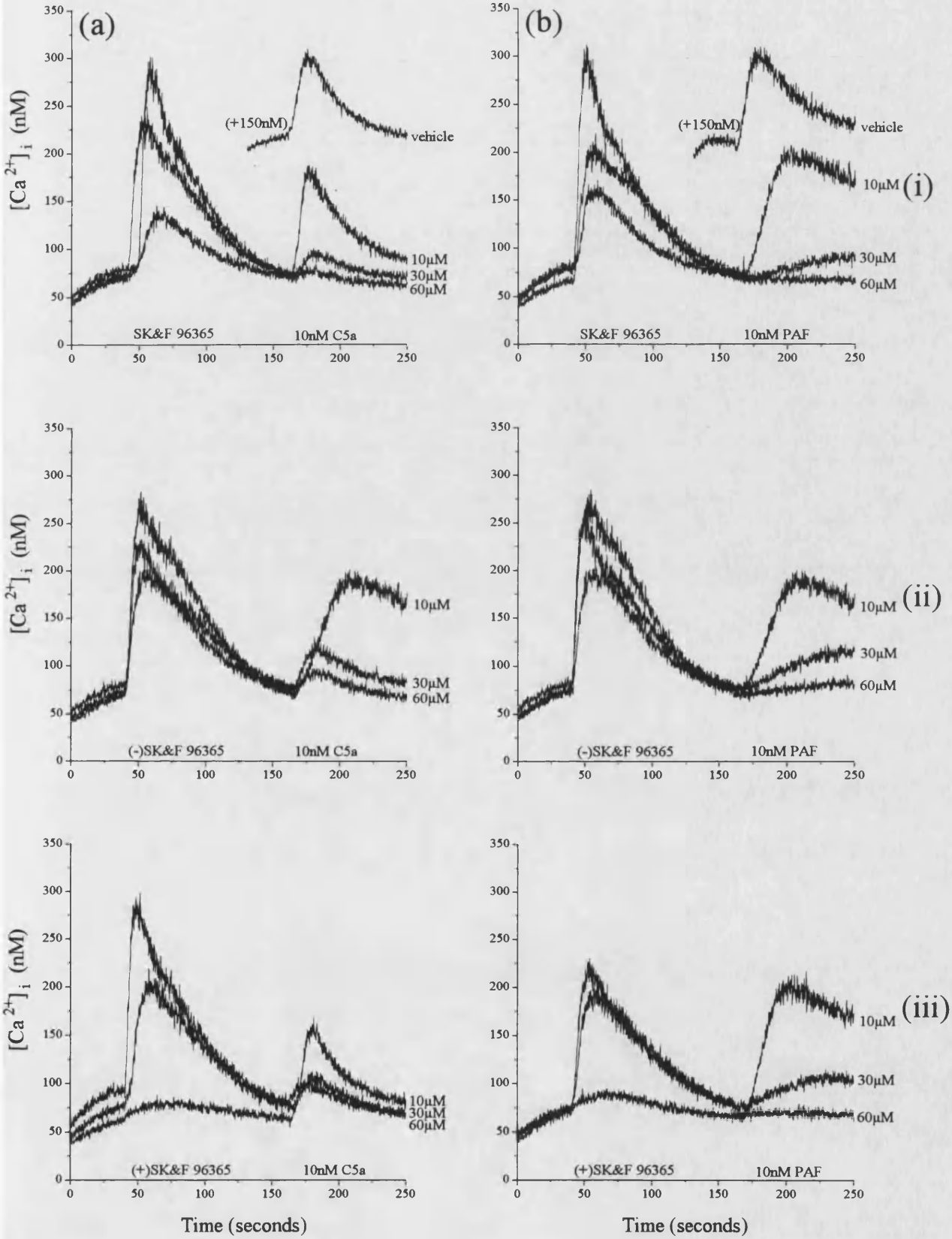

(a)
(b)
(+150nM)
vehicle
10μM
30μM
60μM
(i)
SK&F 96365
10nM C5a
10nM PAF
(-)SK&F 96365
(ii)
(+)SK&F 96365
(iii)
[Ca 2+]i (nM)
Time (seconds)

Figure 5.8

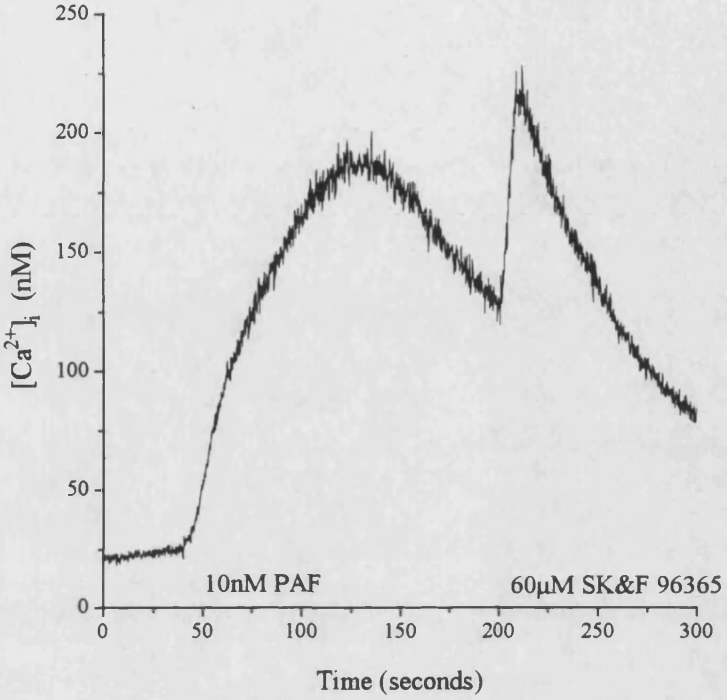


Figure 5.9 Time course of $[Ca^{2+}]_i$ elevation in fura-2-loaded guinea pig eosinophils ($2x10^6$) stimulated with PAF (10nM) then SK&F 96365 (60μM). Trace is representative of one experiment.

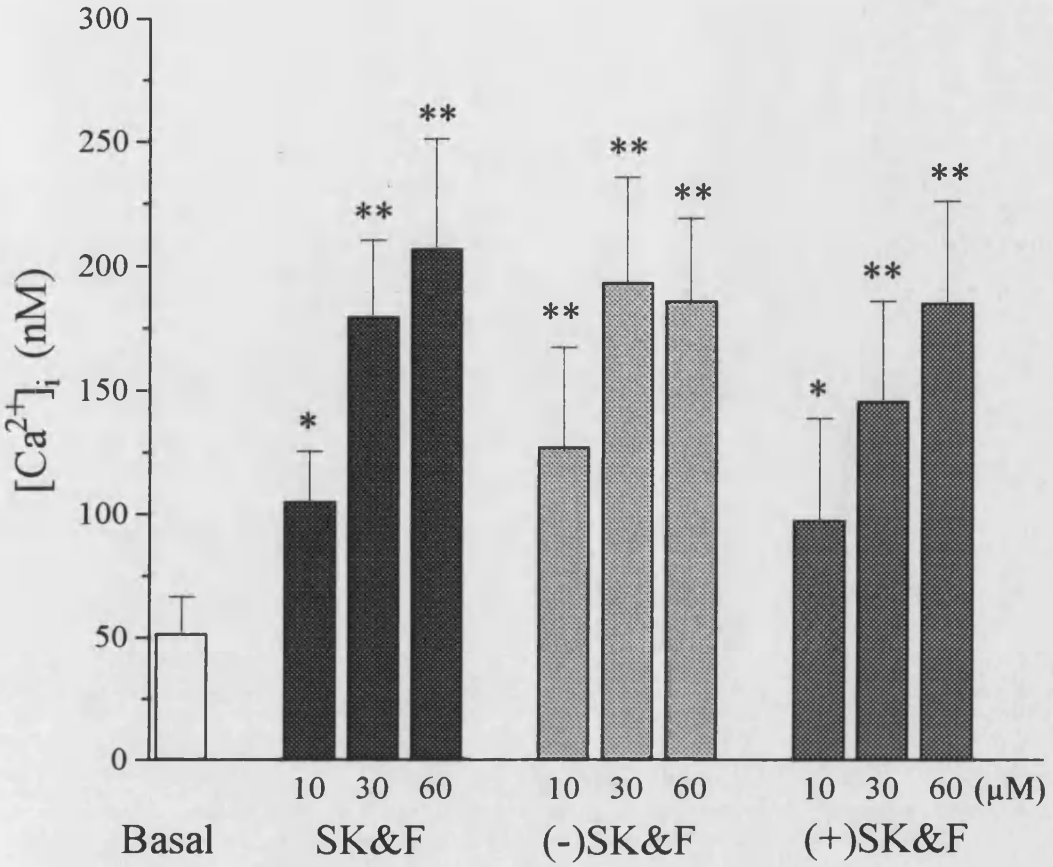


Figure 5.10 $[Ca^{2+}]_i$ levels in fura-2-loaded guinea pig eosinophils either unstimulated (□) or stimulated with SK&F 96365 (10-60μM) (▩), (-)SK&F 96365 (10-100μM) (▒) or (+)SK&F 96365 (10-100μM) (▨). Bars indicate mean±sem maximal $[Ca^{2+}]_i$/2x10^{6}cells for n=4 different eosinophil preparations. Significantly increased $[Ca^{2+}]_i$ elevation compared with basal $[Ca^{2+}]_i$ levels: *, $p<0.05$; **, $p<0.01$.

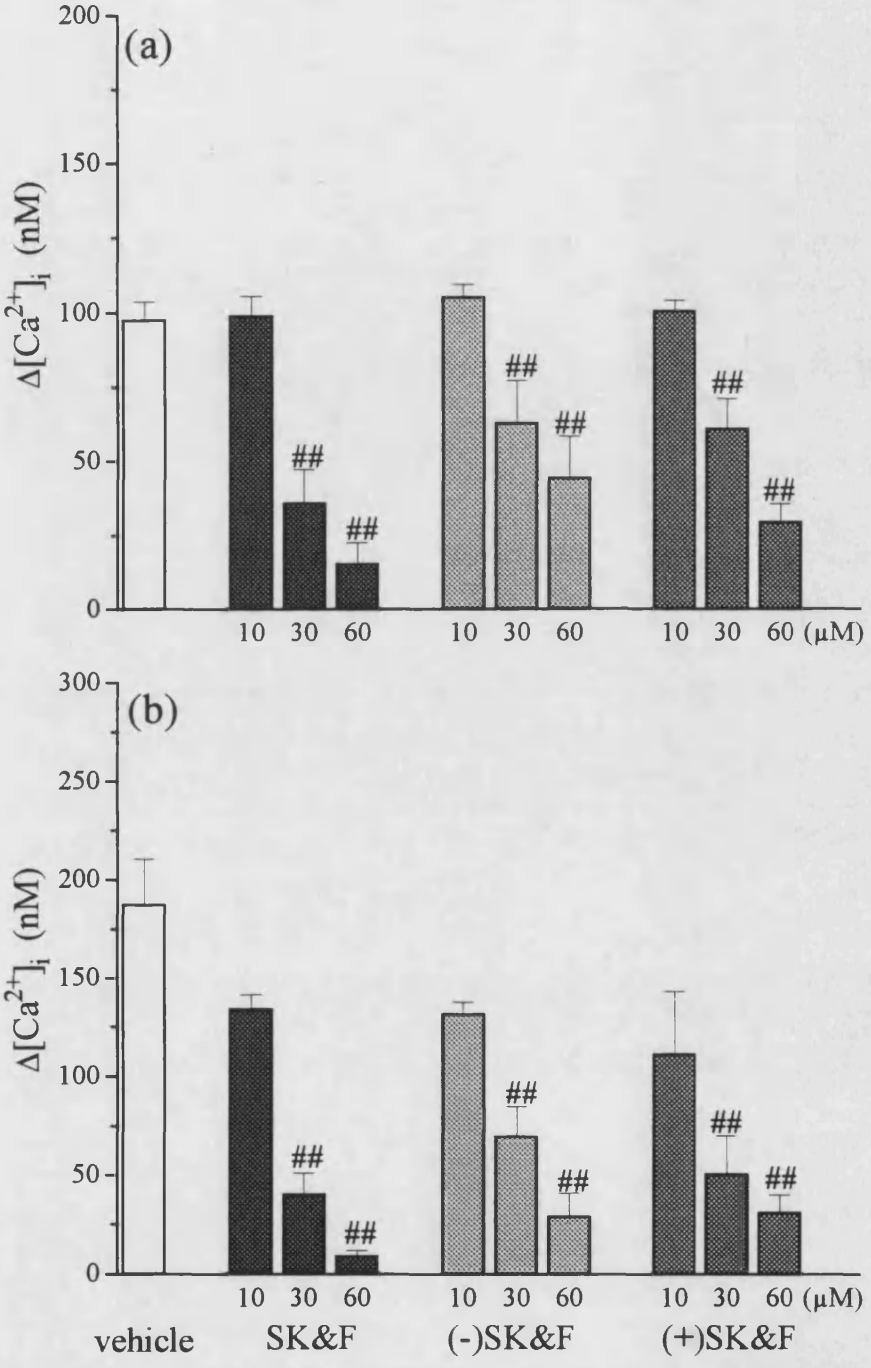


Figure 5.11 Elevation of $[Ca^{2+}]_i$ in fura-2-loaded guinea pig eosinophils stimulated with vehicle (□), SK&F 96365 (10-60μM) (▩), (-)SK&F 96365 (10-100μM) (▒) or (+)SK&F 96365 (10-100μM) (▨) then either (a) C5a (10nM) or (b) PAF (10nM). Bars indicate mean±sem $\Delta[Ca^{2+}]_i/2x10^6$cells for n=3-6 different eosinophil preparations. Significantly reduced $[Ca^{2+}]_i$ elevation compared with vehicle stimulated cells: ##, $p<0.01$.

5.1.6 Effect of the PKC inhibitor, Ro 31-8220/002, on $[Ca^{2+}]_i$ elevation

To investigate the role of PKC in calcium signalling, the elevation of free calcium was monitored in response to suboptimal doses of C5a (30nM) and LTB_4 (30nM) in cells preincubated with Ro 31-8220/002 (0.3-3μM). Interestingly, the inhibitor dose-dependently potentiated the maximal responses as shown in figure 5.12. It can be clearly observed in the LTB_4 biphasic response that it is the second peak of the response that is enhanced. Both C5a and LTB_4 exhibited a significant rise at 3μM Ro 31-8220/002 as indicated in figure 5.13

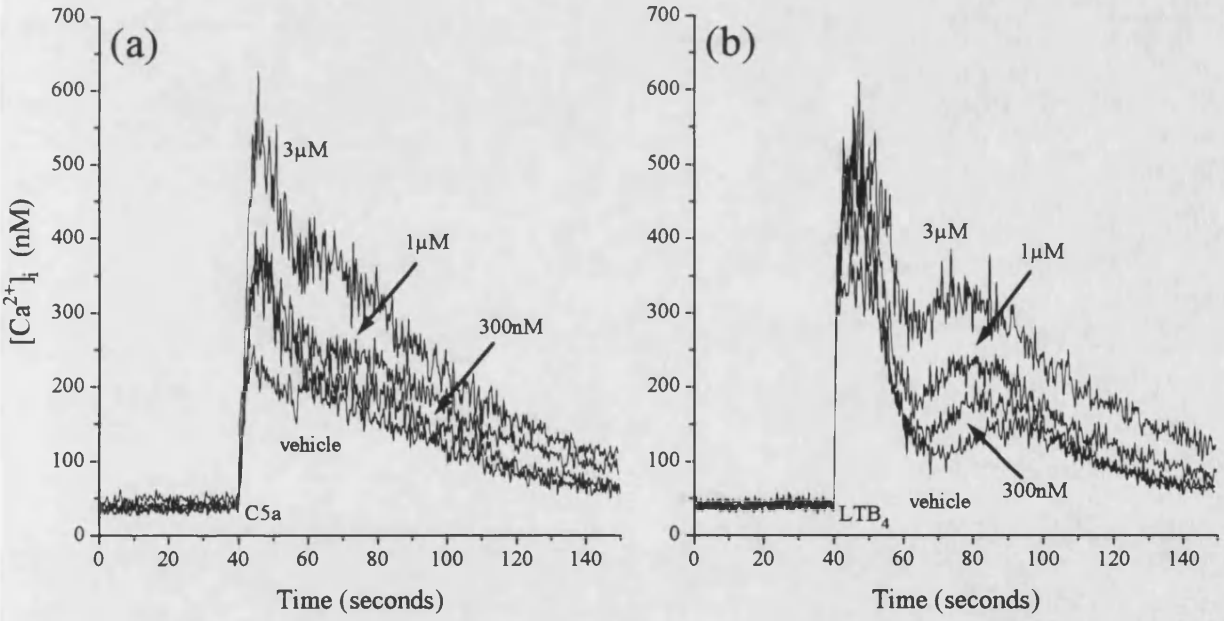


Figure 5.12 Time courses of $[Ca^{2+}]_i$ elevation in fura-2-loaded guinea pig eosinophils ($2x10^6$) pretreated with the PKC selective inhibitor Ro 31-8220/002 (for 20min at 37°C) then stimulated with either (a) C5a (30nM) or (b) LTB_4 (30nM). Traces are representative of 4 different eosinophil preparations.

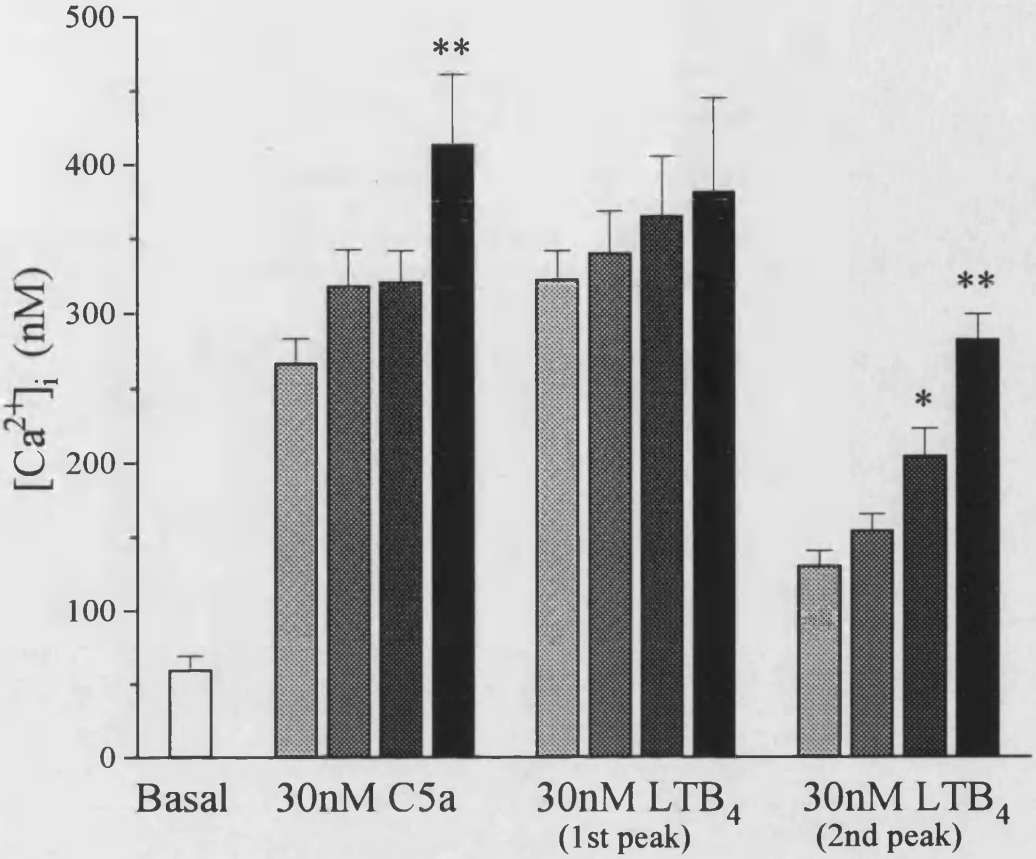


Figure 5.13 Elevation of $[Ca^{2+}]_i$ in fura-2-loaded guinea pig eosinophils pretreated with vehicle (▒), 300nM (▨), 1μM (▩) or 3μM (■) of the PKC selective inhibitor Ro 31-8220/002 (for 20min at 37°C) then stimulated with either C5a (30nM) or LTB_4 (30nM). Basal $[Ca^{2+}]_i$ levels (□). Bars indicate mean±sem maximal $[Ca^{2+}]_i/2x10^6$cells for n=4 different eosinophil preparations. Significantly increased $[Ca^{2+}]_i$ elevations compared with vehicle treated cells: *, $p<0.05$; **, $p<0.01$.

5.1.7 Effect of the tyrosine kinase inhibitors genistein, erbstatin, herbimycin A and tyrphostin A-47

The role of tyrosine kinase on calcium signalling was also investigated. For similar reasons to those for the H_2O_2 assay, before any experiments were done the effect of each inhibitor on fluorescence was first investigated. Figure 5.14 shows that erbstatin (100μM) and tyrphostin A-47 (10μM) had significant effects on the fura-2 fluorescence signal. Similarly erbstatin (100μM), tyrphostin A-47 (100μM) and

genistein (300μM) had significant effects on fluo-3. Herbimycin A was the only inhibitor that did not affect fura-2 and genistein (100μM) was safe to use with fluo-3 and these agents were thus investigated on $[Ca^{2+}]_i$ in guinea pig eosinophils.

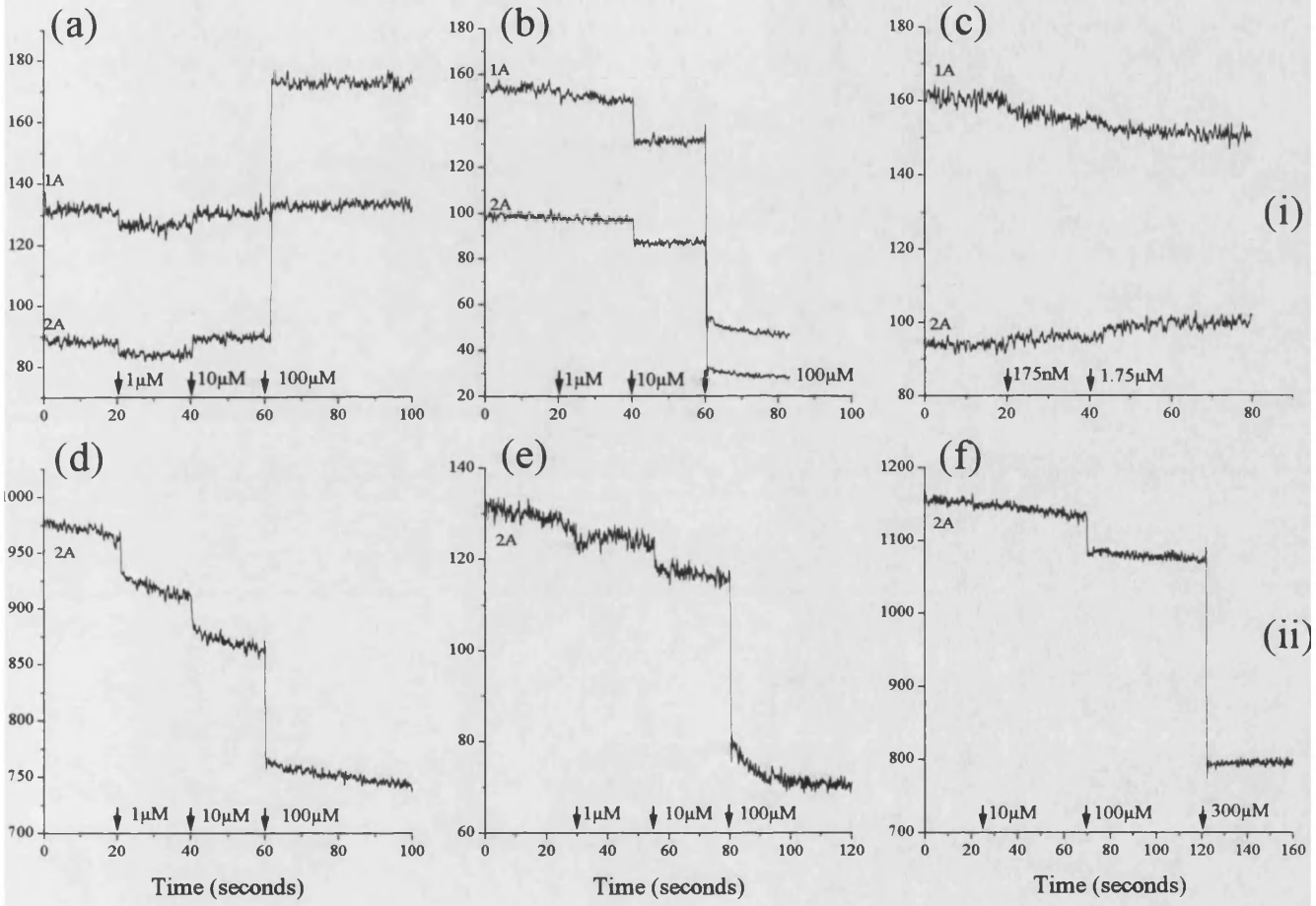


Figure 5.14 Effect of the fluorescent properties of different specific tyrosine kinase inhibitors on (i) free fura-2 (Ex1=340nm, Ex2=280nm and Em=510nm) or (ii) free fluo-3 (Ex=510nm and Em=526nm): (a&d) erbstatin (1-100μM); (b&e) tyrphostin A-47 (1-100μM); (c) herbimycin A (0.175-1.75μM) and (f) genistein (10-300μM).

Herbimycin A or genistein had no effect on $[Ca^{2+}]_i$ elevation in guinea pig eosinophils. Figure 5.15 shows the identical traces obtained in C5a (10nM) or PAF (10nM) stimulated eosinophils incubated with either vehicle or herbimycin A (1μM) (the data for genistein is not shown).

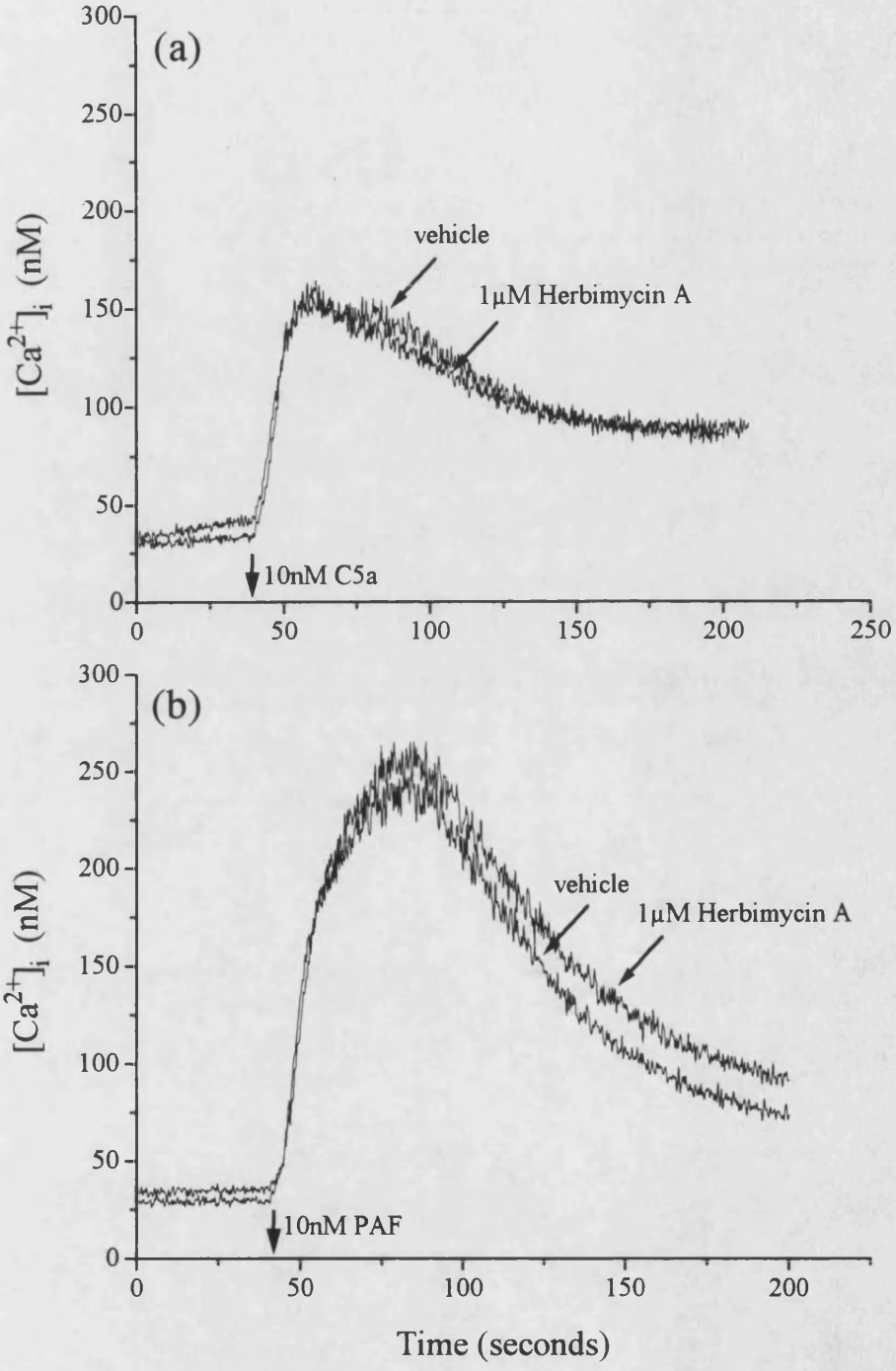


Figure 5.15 Time courses of $[Ca^{2+}]_i$ elevation in fura-2-loaded guinea pig eosinophils ($2x10^6$) pretreated with herbimycin A (1μM) for 20min at 37°C before stimulation with (a) C5a (10nM) or (b) PAF (10nM). Traces are representative of 2 experiments.

5.2 Human eosinophil

5.2.1 Effect of added agonists including chemokines and the cytokine IL-5

The human eosinophil preparations had a basal $[Ca^{2+}]_i$ of 75.7±5.3nM (n=8). Upon stimulation with RANTES, MIP-1α, MCP-1 and MCP-3 (1-100nM) all the chemokines produced characteristic responses (figure 5.16). After addition of the agonist, elevations in $[Ca^{2+}]_i$ were rapid (within the first 2sec following addition) with a gradual decrease back to near basal levels over the next 20-30sec. RANTES, MIP-1α and MCP-3 produced a dose-related increase in $[Ca^{2+}]_i$ above basal levels that were significant at 10nM and 100nM for each agonist. The MCP-1 induced elevation was only significant at 100nM as shown in figure 5.17(b). Neither MIP-1β nor IL-5 could elevate calcium in human eosinophils (figure 5.16). The lack of activity of IL-5 was in contrast to its very potent effect in the H_2O_2 assay (figures 3.21 and 3.22). In addition to the human cytokines, the agonists C5a and PAF were also tested for their ability to elevate $[Ca^{2+}]_i$ in human eosinophils. As figure 5.17(a) demonstrates both C5a and PAF were potent inducers of $[Ca^{2+}]_i$ elevation. Unlike the effect on guinea pig eosinophils where these agonists were approximately equipotent, in human eosinophils C5a caused $[Ca^{2+}]_i$ elevations at concentrations as low as 30pM and significance was acheived at 300pM. LTB_4 was not tested on human eosinophils.

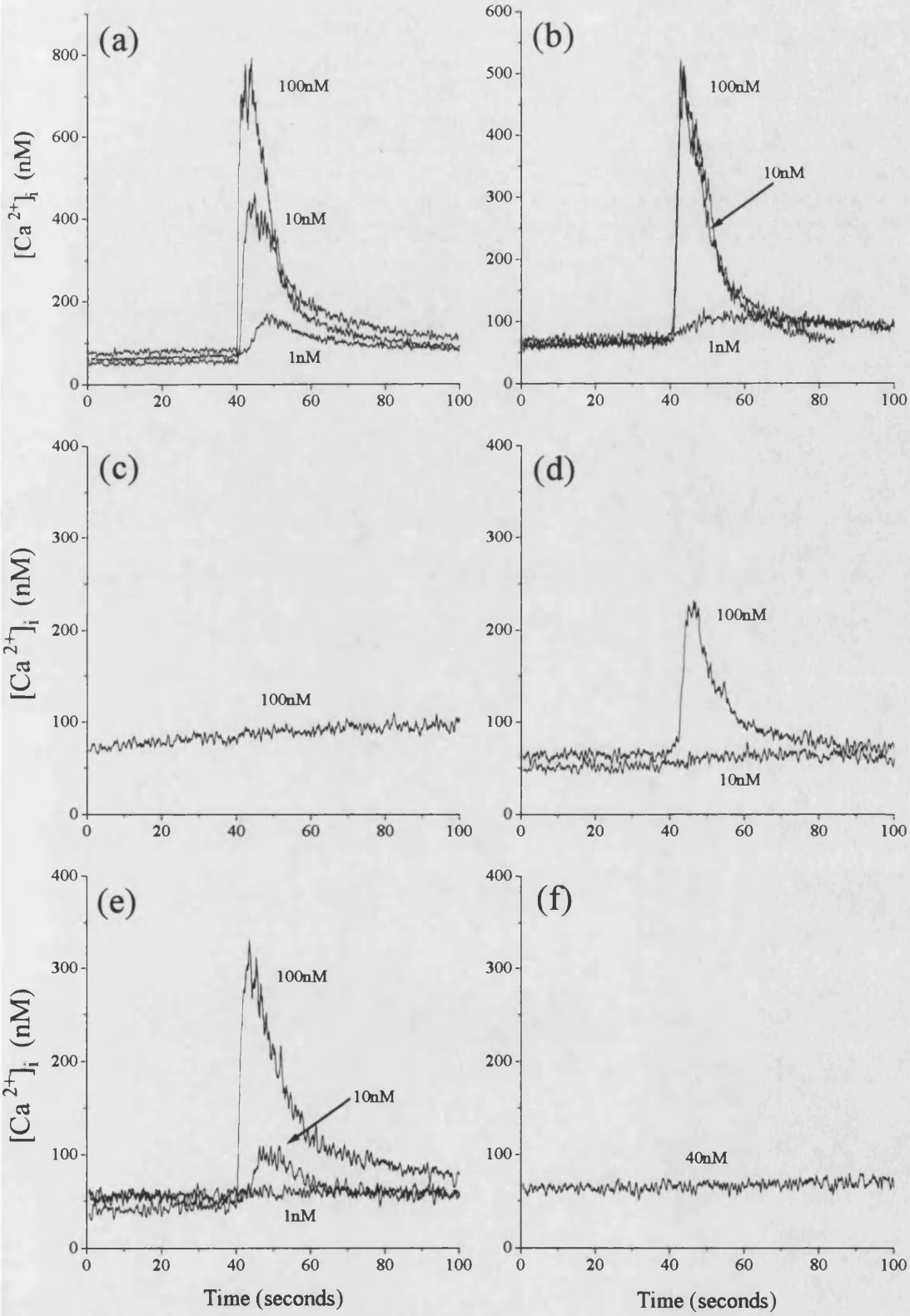


Figure 5.16 Time courses of $[Ca^{2+}]_i$ elevation in fura-2-loaded human eosinophils ($1x10^6$) stimulated with (a) RANTES (1-100nM), (b) MIP-1α (1-100nM), (c) MIP-1β (100nM), (d) MCP-1 (10-100nM), (e) MCP-3 (1-100nM) or (f) IL-5 (40nM). Traces are representative of 2-6 experiments.

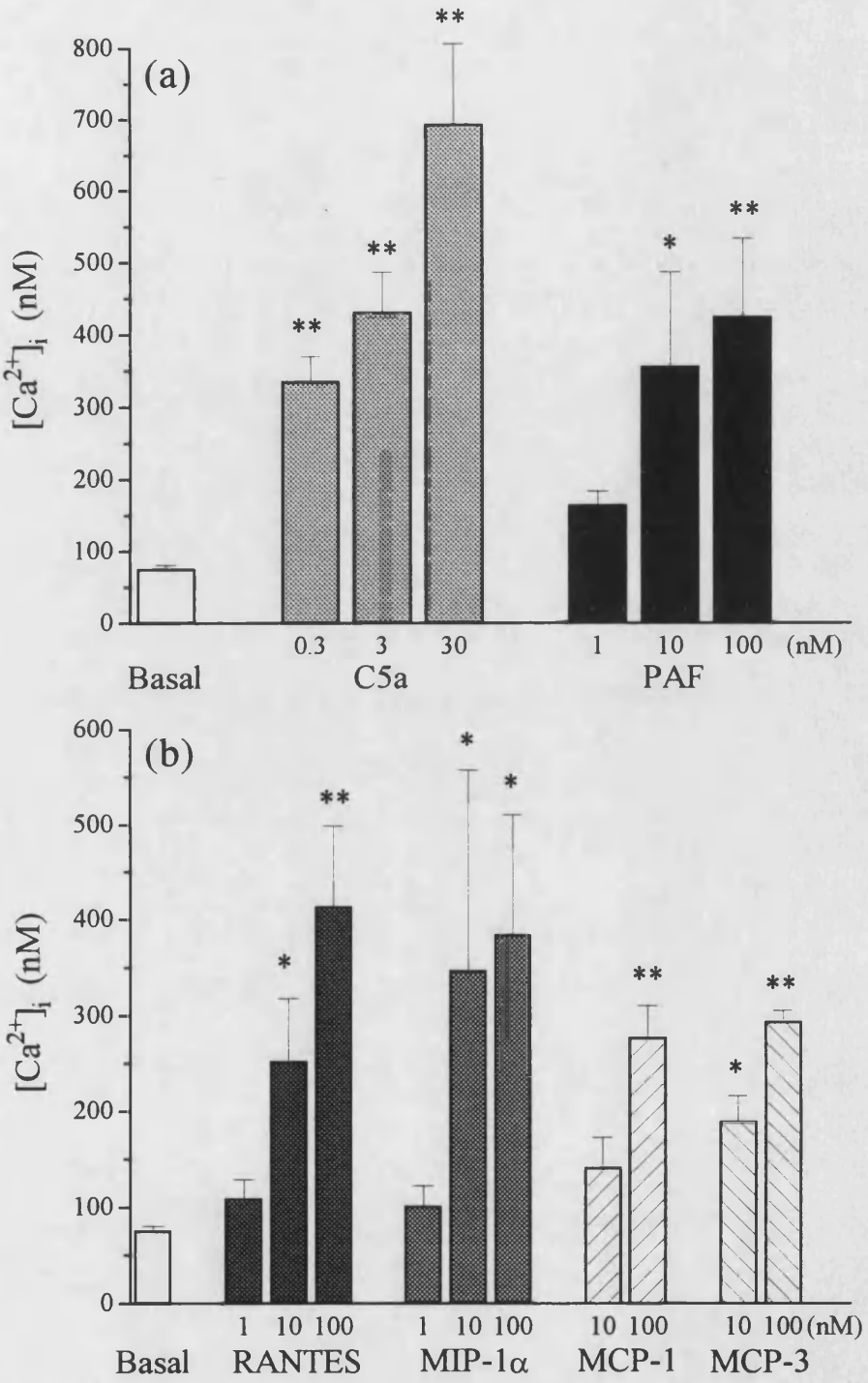


Figure 5.17 $[Ca^{2+}]_i$ levels in fura-2-loaded human eosinophils (a) stimulated with the agonists C5a (0.3-30nM)(▒) or PAF (1-100nM)(■) or (b) stimulated with the chemokines RANTES (1-100nM)(▩), MIP-1α (1-100nM)(▨), MCP-1 (10-100nM)(▨), MCP-3 (1-100nM)(▧). Basal $[Ca^{2+}]_i$ levels (□). Bars indicate mean±sem maximal $[Ca^{2+}]_i/1x10^6$cells for n=3-8 different eosinophil preparations. Significantly increased $[Ca^{2+}]_i$ elevation compared with basal levels: *, $p<0.05$; **, $p<0.01$.

5.2.2 Desensitisation experiments between RANTES and MCP-3

Previously it has been reported using desensitisation experiments that a RANTES specific receptor and a MIP-1α/RANTES receptor exists (44,236). These experiments have been successfully repeated (data not shown). I then further investigated whether the chemokines MCP-1 and MCP-3 used a common chemokine receptor or used specfic receptors of their own. Figure 5.18(a) demonstrates that desensitisation was observed when human eosinophils were stimulated with RANTES followed by MCP-3, but not vice versa. This indicates MCP-3 responses are mediated by the RANTES receptor and these data are in agreement with the only previous study (236). MCP-1 receptors have been reported not to exist on eosinophils (44) however a significant response to MCP-1 (100nM) on human eosinophils was detected (figure 5.16). Figure 5.18(b) shows that MCP-3 desensitises the response elicited by MCP-1 but not vice versa. MCP-1 is not acting via the RANTES receptor. This is ruled out by the activity of the chemokine MCP-3.

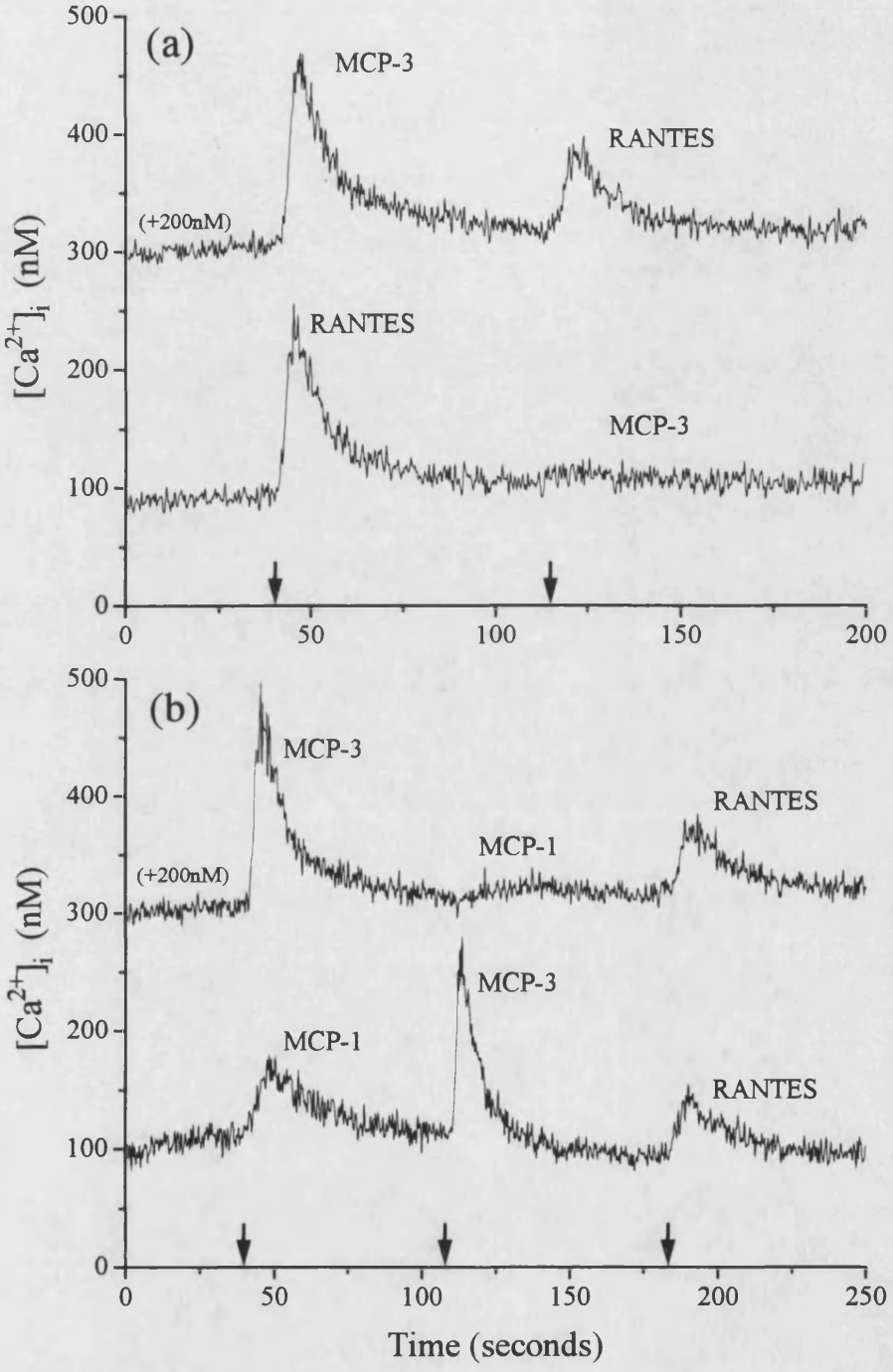


Figure 5.18 Time courses of $[Ca^{2+}]_i$ elevation cross-desensitisation experiments in fura-2-loaded human eosinophils ($1x10^6$) stimulated with (a) MCP-3/RANTES (10nM) or (b) MCP-1/MCP-3/RANTES (10nM). Traces are representative of 2 experiments.

5.2.3 Effect of EGTA and EDTA on $[Ca^{2+}]_i$

To ascertain the effect of extracellular calcium on human eosinophils $[Ca^{2+}]_i$, preliminary experiments were performed using EGTA and EDTA (4mM). Figure 5.19 shows that when eosinophils were stimulated with RANTES (10nM) in the presence of EGTA, the response was attenuated by around 80%. The effect of EDTA was more minimal with only a partial reduction (25%) of the signal. Both these results again indicate an influx component in the signalling system.

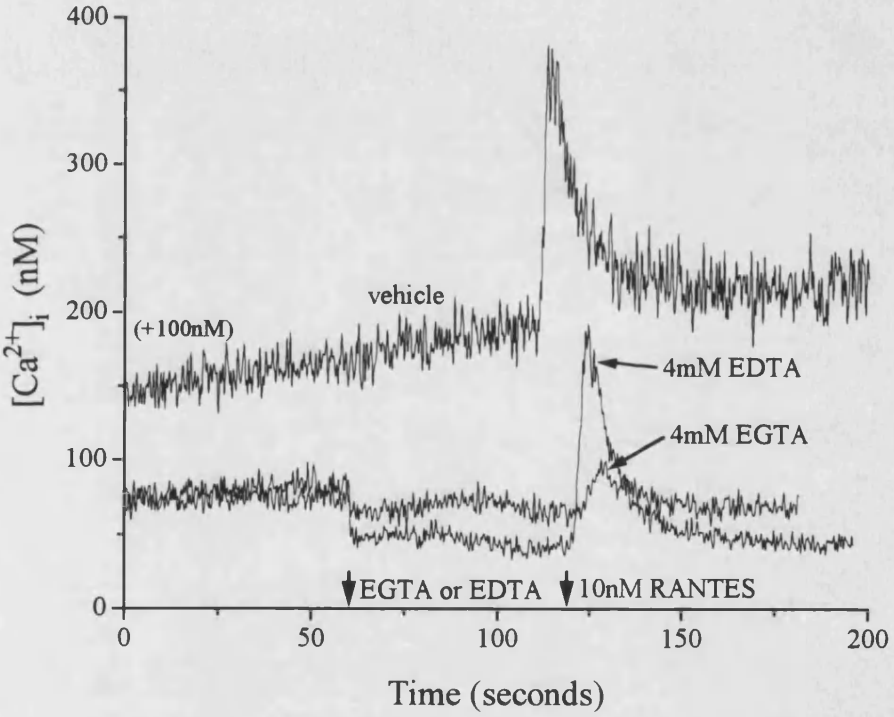


Figure 5.19 Time courses of $[Ca^{2+}]_i$ elevation in fura-2-loaded human eosinophils ($1x10^6$) in the presence of vehicle, EGTA (4mM) or EDTA (4mM) then stimulated with RANTES (10nM). Traces are representative of 2 experiments.

5.2.4 Effect of the ROCC blocker, SK&F 96365, on $[Ca^{2+}]_i$

On guinea pig eosinophils, SK&F 96365 appeared to have an 'agonistic' effect in $[Ca^{2+}]_i$ elevation. Thus the compound was tested for its effect on human eosinophils in $[Ca^{2+}]_i$ elevation. As in the case of H_2O_2 production, the effects of SK&F 96365 on human eosinophils in $[Ca^{2+}]_i$ elevations were very different to the case of guinea pig eosinophils. Figures 5.20 and 5.21 illustrate that SK&F 96365 (10-60μM) did not affect $[Ca^{2+}]_i$ elevation in human eosinophils, however these concentrations all caused a significant $[Ca^{2+}]_i$ elevations in guinea pig eosinophils (figures 5.8 and 5.10). Only at the highest dose tested (100μM), was a small significant rise in $[Ca^{2+}]_i$ detected. Figure 5.20 shows that SK&F 96365 (10-100μM) dose-dependently inhibited the subsequent response to the added agonists PAF (10nM) and RANTES (100nM). Figure 5.22 shows the inhibition to be significant by analysing pooled data from several experiments. In addition the agonist C5a (300pM) was tested.

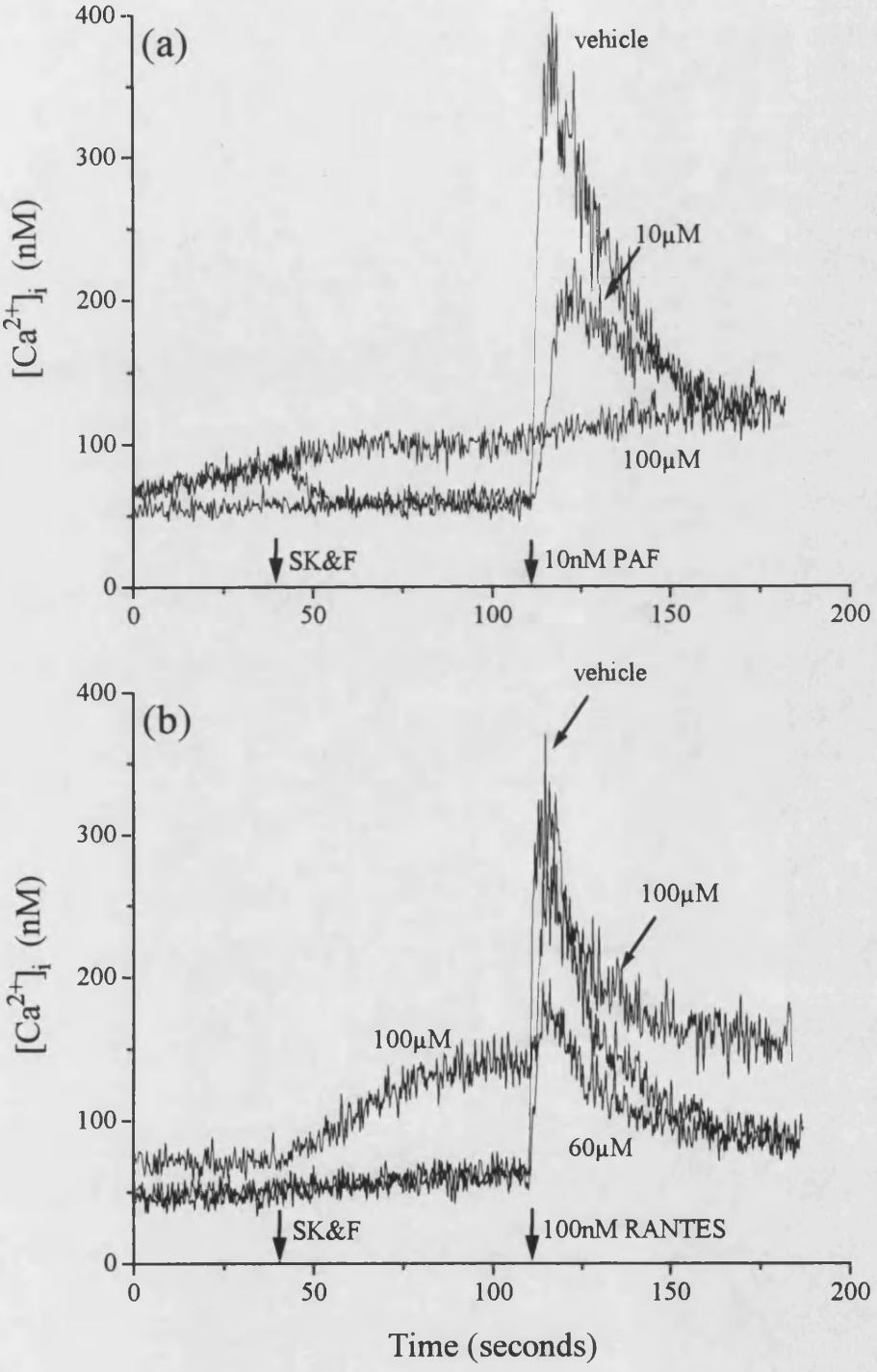


Figure 5.20 Time courses of $[Ca^{2+}]_i$ elevation in fura-2-loaded human eosinophils ($1x10^6$cells) pretreated with SK&F 96365 (10-100μM) added at 40 seconds then either (a) PAF (10nM) or (b) RANTES (100nM) added at 110 seconds. Traces are representative of 3-5 experiments.

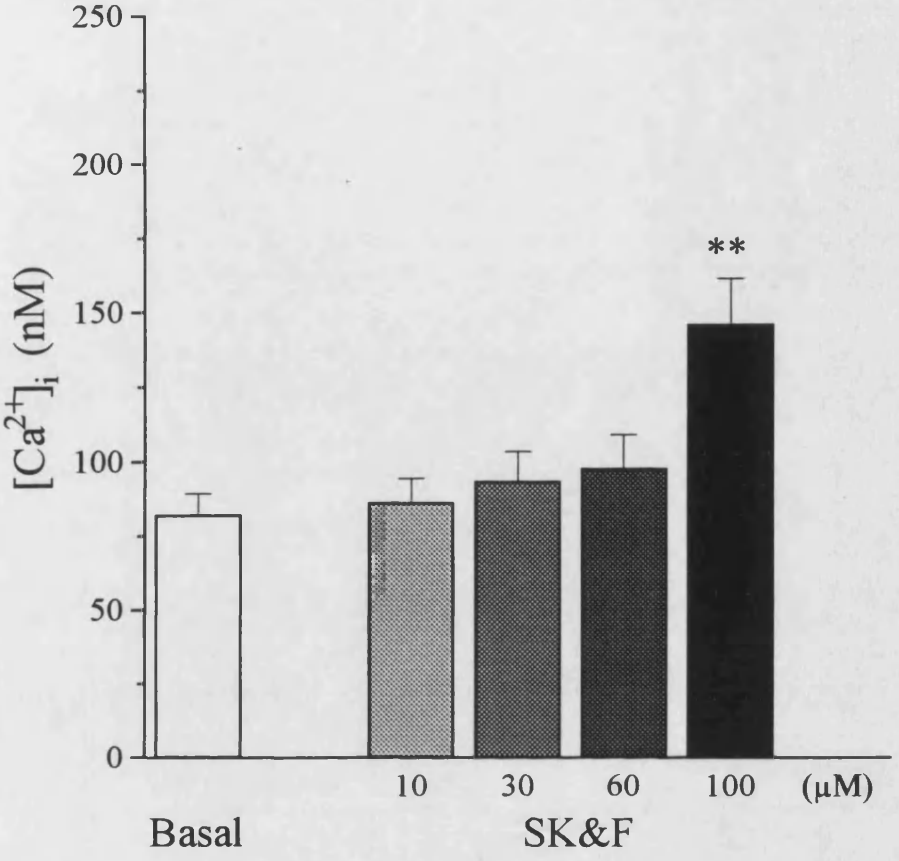


Figure 5.21 $[Ca^{2+}]_i$ levels in fura-2-loaded eosinophils after the addition of SK&F 96365: 10μM (▒); 30μM (▒); 60μM (▒); 100μM (■). Basal $[Ca^{2+}]_i$ levels (□). Bars indicate mean±sem maximal $[Ca^{2+}]_i/1x10^6$ cells for n=5 different eosinophil preparations. Significantly increased $[Ca^{2+}]_i$ elevation compared with basal $[Ca^{2+}]_i$ levels: **, $p<0.01$.

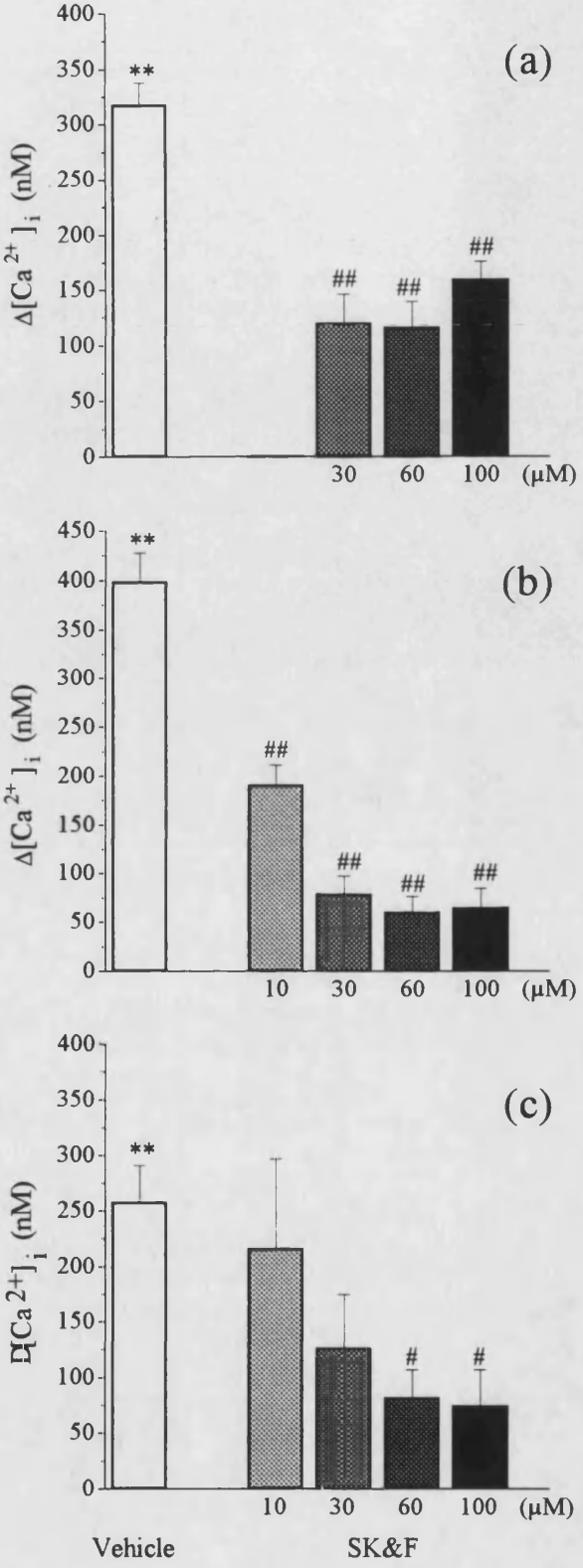


Figure 5.22 Elevation of $[Ca^{2+}]_i$ in fura-2-loaded eosinophils after the addition of SK&F 96365: vehicle (□); 10μM (▒); 30μM (▓); 60μM (▓); 100μM (■) then stimulated with either (a) C5a (300pM), (b) PAF (10nM) or (c) RANTES (100nM). Bars indicate mean±sem $\Delta[Ca^{2+}]_i/1x10^6$ cells for n=3 different eosinophil preparations. Significantly increased $[Ca^{2+}]_i$ elevation compared with basal $[Ca^{2+}]_i$ levels. Significantly reduced $[Ca^{2+}]_i$ elevation compared with vehicle stimulated cells: #, $p<0.05$; ##, $p<0.01$.

5.2.5 Effect of the selective PI 3-kinase inhibitor, wortmannin, in $[Ca^{2+}]_i$

The specific PI 3-kinase inhibitor, wortmannin, greatly affected H_2O_2 production from both guinea pig and human eosinophils. Its effect was thus tested on $[Ca^{2+}]_i$ elevation in human eosinophils. Figure 5.23 shows that the addition of wortmannin (3-30nM) did not alter the $[Ca^{2+}]_i$ in human eosinophils. After a 10min incubation at 37°C the chemokine RANTES (100nM), the response which was significantly inhibited in the H_2O_2 assay, was added to the cuvette.

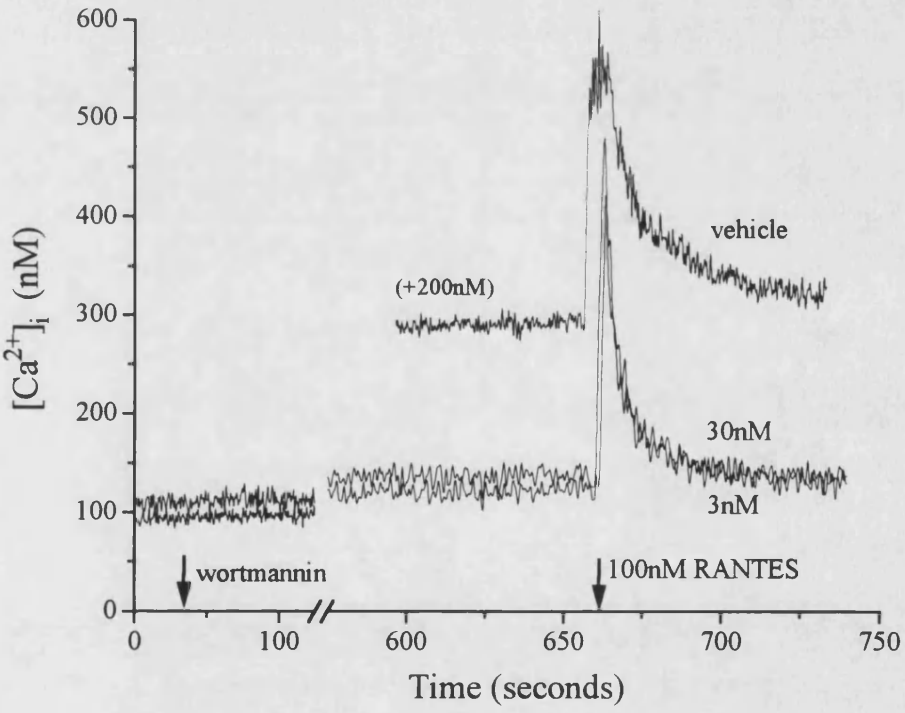


Figure 5.23 Time courses of $[Ca^{2+}]_i$ elevation in fura-2-loaded human eosinophils ($1x10^6$cells) pretreated with vehicle or the PI 3-kinase specific inhibitor wortmannin (for 10min at 37°C) then stimulated with RANTES (100nM). The control response (without wortmannin) is shifted by 200nm for clarity. Traces are from a single experiment.

As illustrated in figure 5.23, both vehicle and wortmannin treated eosinophils

elicited identical $[Ca^{2+}]_i$ traces, suggesting PI 3-kinase did not affect the calcium signalling pathway. In addition, it demonstrates that wortmannin did not affect the agonist receptors or was toxic to the cells.

5.3 Eol-1s and Eol-3s

5.3.1 Effect of added agonists

The human eosinophilic cell lines Eol-1 and Eol-3 were tested for their ability to elevate $[Ca^{2+}]_i$ in response to various added agonists and chemokines. Both unmatured and cells matured with butyric acid were tested. From Leishman's stained cytospins of Eol-1 and Eol-3s of unmatured and matured cells, there was no visible differentiation from mononuclear blast-like cells towards the granulocytic eosinophil type cells. Figure 5.24 shows the response of unmatured and matured Eol-1s and Eol-3s when stimulated with the agonist PAF (100nM) and the chemokine RANTES (1-100nM). It appears that Eol-1s are generally more responsive to agonists than Eol-3s. There was no difference between unmatured and matured Eol-1s; unmatured cells were thus used for most of the experiments. In contrast Eol-3s became more responsive as they matured with dubutyryl cAMP. However this applied to all the agonists tested and not those that were specific to human eosinophils. Figure 5.25 shows the pooled data for all the agonists tested. PAF (10-100nM), RANTES (10-100nM) and MCP-1 (0.1-100nM) dose-dependently elevated $[Ca^{2+}]_i$ in unmatured Eol-1s. The same was true for two-day dibutyryl cAMP (0.2mM) matured Eol-1s. The maximal elevation of $[Ca^{2+}]_i$ in unmatured Eol-3s was considerably lower than that of Eol-1s.

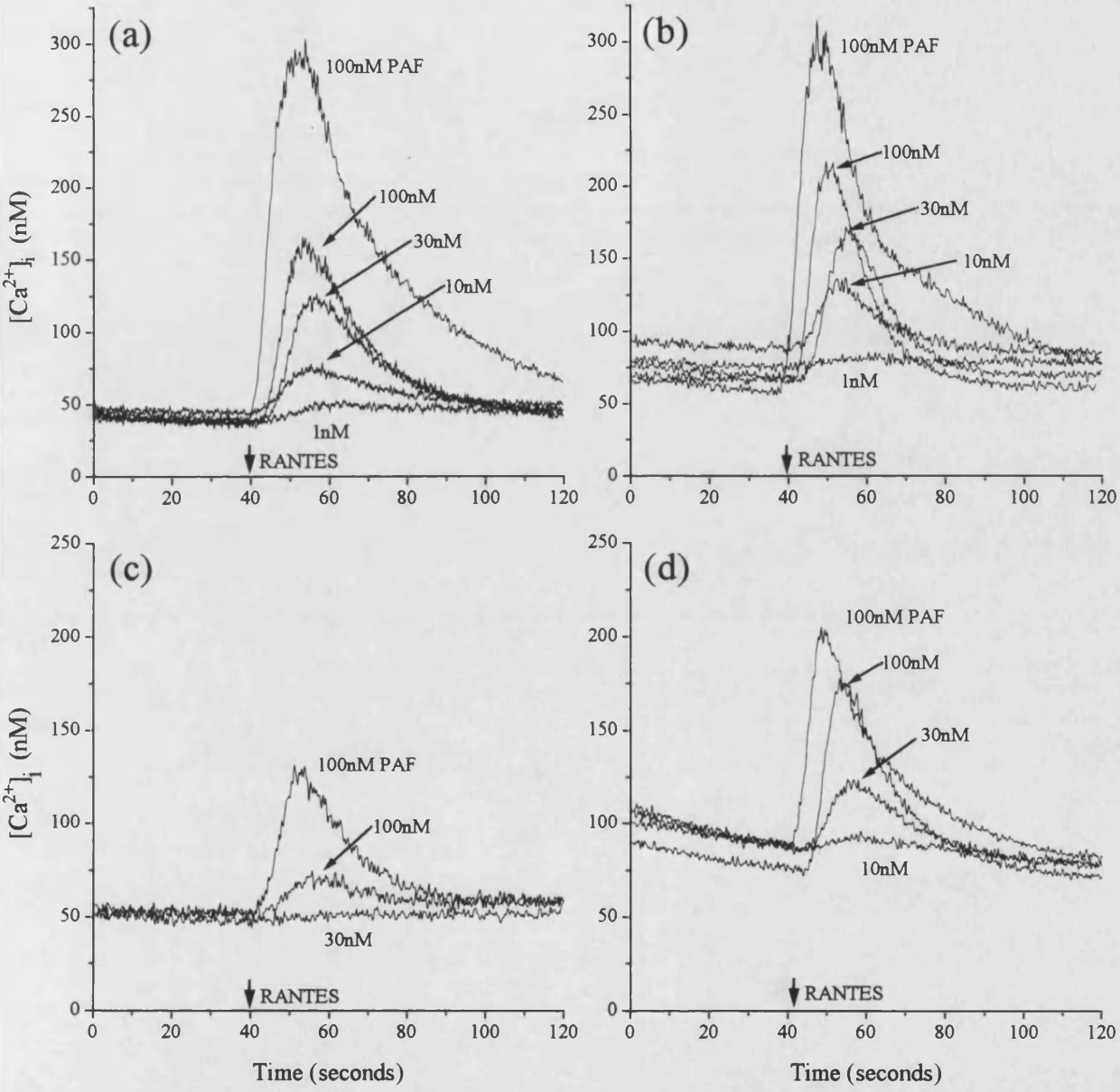


Figure 5.24 Time courses of $[Ca^{2+}]_i$ elevation in fura-2-loaded Eol-1s and Eol-3s ($2x10^6$cells) stimulated at 40 seconds with either PAF (100nM) or RANTES (1-100nM) in (a) unmatured Eol-1s; (b) two-day dibutyryl cAMP (0.2mM) matured Eol-1s; (c) unmatured Eol-3s or (d) two-day dibutyryl cAMP (0.2mM) matured Eol-3s. Traces are representative of 1-6 representative experiments.

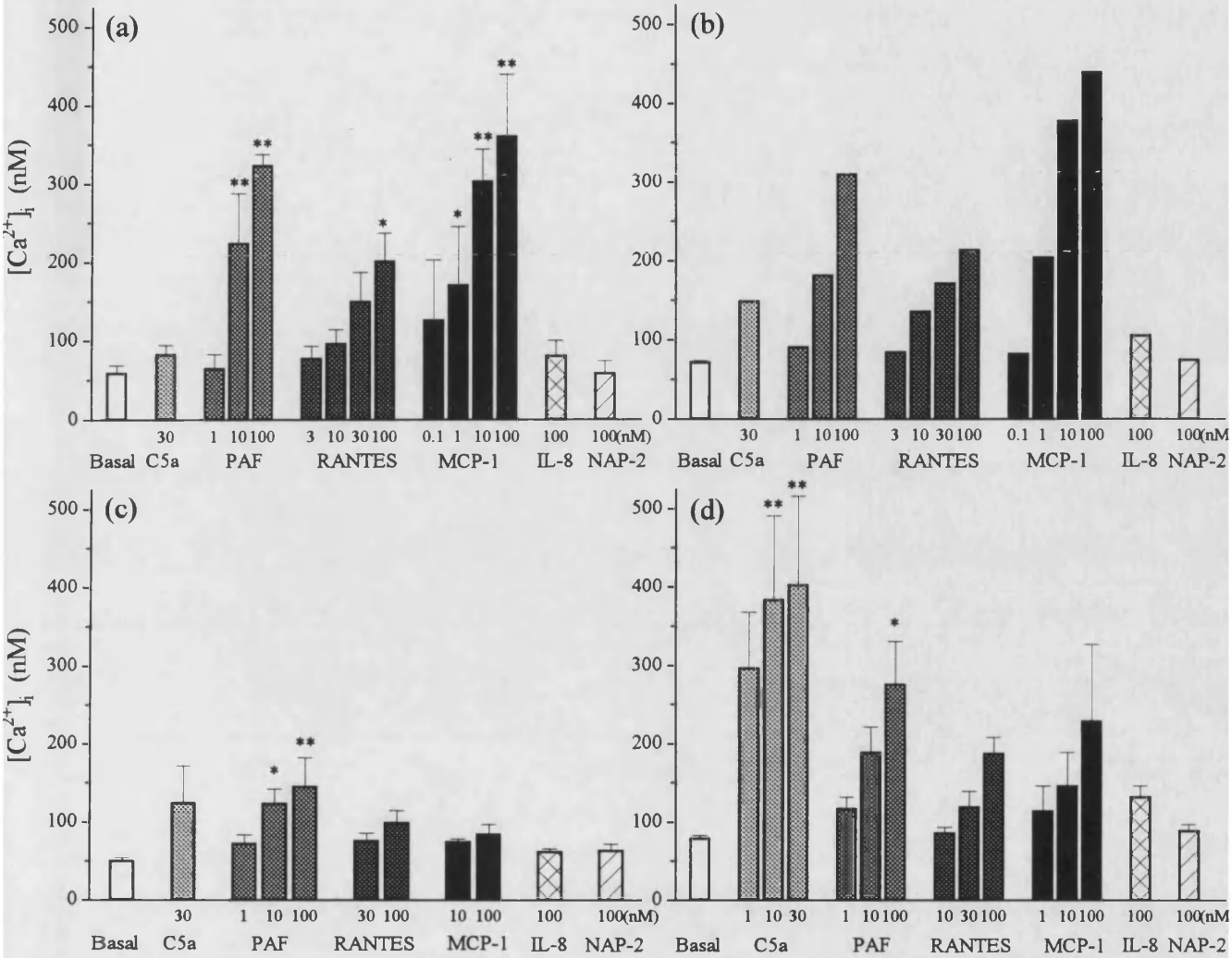


Figure 5.25 $[Ca^{2+}]_i$ levels in fura-2-loaded Eol-1s and Eol-3s stimulated with C5a (1-30nM)(▒), PAF (1-100nM)(▒), RANTES (3-100nM)(▒), MCP-1 (0.1-100nM)(■), IL-8 (100nM)(⊠) or NAP-2 (100nM)(▨) in either (a) unmatured Eol-1s; (b) two-day dibutyryl cAMP (0.2mM) matured Eol-1s; (c) unmatured Eol-3s or (d) two-day dibutyryl cAMP (0.2mM) matured Eol-3s. Basal $[Ca^{2+}]_i$ levels (□). Bars indicate mean±sem maximal $[Ca^{2+}]_i$/2x10^{6}cells for n=3-6 different eosinophil preparations (except for two-day dibutyryl cAMP (0.2mM) matured Eol-1s where n=1). Significantly increased $[Ca^{2+}]_i$ elevation compared with basal $[Ca^{2+}]_i$ levels: *, $p<0.05$; **, $p<0.01$.

Unmatured Eol-3s did elevate $[Ca^{2+}]_i$ in response to C5a (30nM), PAF (10-100nM), RANTES (30-100nM) and MCP-1 (10-100nM). C5a (1-30nM), PAF (1-

100nM), RANTES (30-100nM), MCP-1 (1-100nM) and IL-8 (100nM) elevated $[Ca^{2+}]_i$ in two-day dibutyryl cAMP (0.2mM) matured Eol-3s. NAP-2 (100nM) did not affect the $[Ca^{2+}]_i$ elevation in any of the cells tested.

5.3.2 Effect of the specific tyrosine kinase inhibitors genistein and herbimycin A on unmatured Eol-1s

To investigate the role of tyrosine kinase in the eosinophilic cell line, unmatured Eol-1s were incubated overnight with vehicle, genistein (30μM) and herbimycin A (875nM), then loaded with fluo-3. Figure 5.26 shows the elevation of $[Ca^{2+}]_i$ (calculated using the difference between fully bound and fully dissociated fluo-3 with calcium from a single excitation and single emission trace) in Eol-1s elicited by PAF (100nM), RANTES (30nM) and MCP-1 (10nM) in the presence of genistein and herbimycin A. It can be seen that the inhibitors did not significantly effect the $[Ca^{2+}]_i$ elevations to any of the agonists tested suggesting PAF, RANTES and MCP-1 did not act via tyrosine kinase linked receptors.

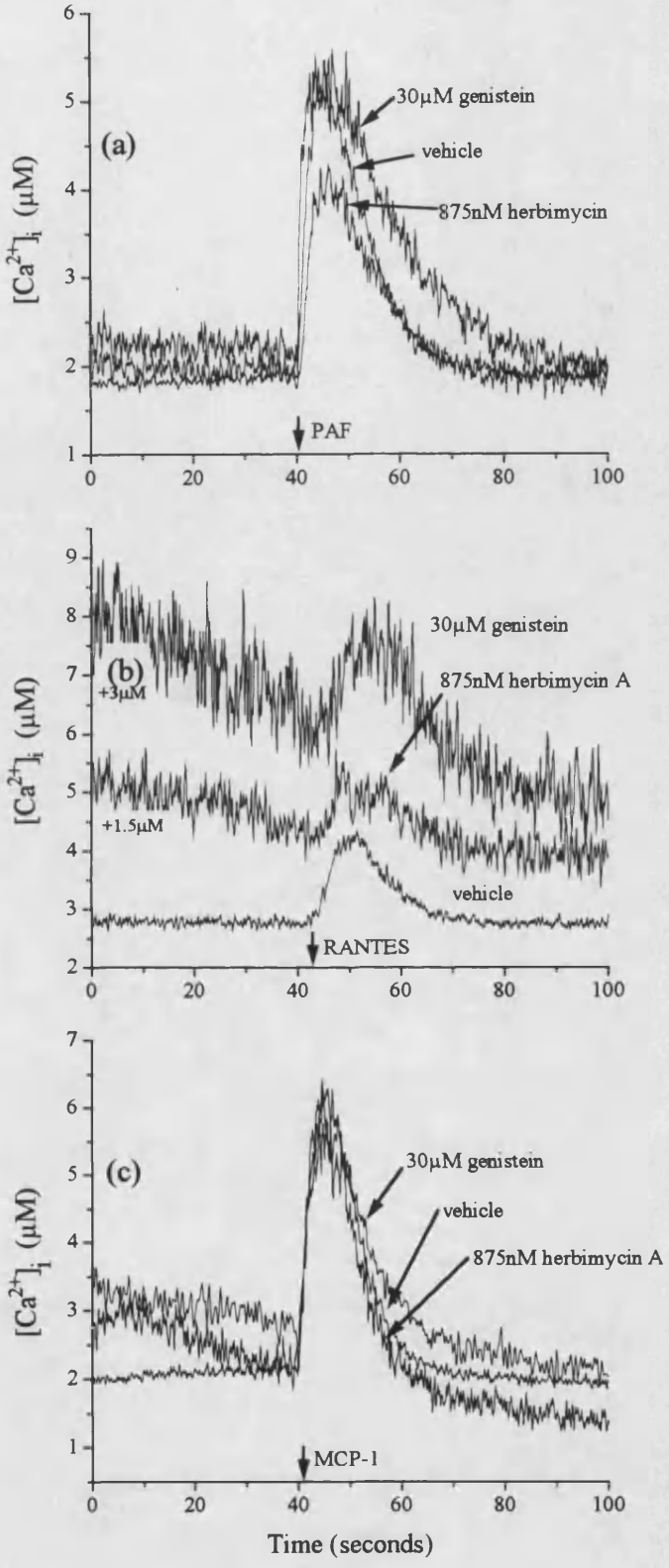
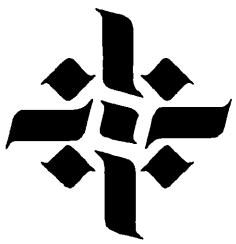


Figure 5.26 Time courses of $[Ca^{2+}]_i$ elevation in fura-2 loaded unmatured Eol-1s ($2x10^6$cells) preincubated with vehicle or the specific tyrosine kinase inhibitors genistein (30μM) or herbimycin A (875nM) (overnight at 37°C) then stimulated with either (a) PAF (100nM), (b) RANTES (30nM) or (c) MCP-1 (10nM). Traces are representative of 2 experiments.

5.4 Summary of $[Ca^{2+}]_i$ experiments

- Guinea pig eosinophils, human eosinophils and eosinophilic Eols can be stimulated to elevate $[Ca^{2+}]_i$.
- Order of potency for $[Ca^{2+}]_i$ elevation in guinea pig eosinophils: LTB_4 > PAF > C5a.
- Order of potency for $[Ca^{2+}]_i$ elevation in human eosinophils: C5a > PAF > RANTES, MIP-1α > MCP-3 > MCP-1.
- Unmatured and matured Eol-1s were equally responsive to added agonists and the order of potency for $[Ca^{2+}]_i$: MCP-1 > RANTES=PAF > C5a > IL-8.
- Matured Eol-3s were more responsive to added agonists than unmatured Eol-3s and the order of potency for $[Ca^{2+}]_i$: C5a > PAF > MCP-1=RANTES > IL-8.
- fMLP, IL-5, IL-8 and NAP-2 were unable to elicit $[Ca^{2+}]_i$ elevation in guinea pig eosinophils.
- IL-5, IL-8 and NAP-2 were unable to elicit $[Ca^{2+}]_i$ elevation in human eosinophils.
- Human eosinophils were found to exhibit a MIP-1α/RANTES receptor and a MCP-3/RANTES receptor.
- Guinea pig and human eosinophils required extracellular calcium to enable normal $[Ca^{2+}]_i$ elevation to added agonist. This avoids intracellular calcium store depletion.
- Nickel and manganese experiments both suggested a small calcium influx component in guinea pig eosinophils.

- SK&F 96365 and its enantiomers concentration-dependently elevated $[Ca^{2+}]_i$ levels in guinea pig eosinophils and concentration-dependently inhibited $[Ca^{2+}]_i$ elevation to the subsequent added agonist. However, the added agonist did not desensitise the effect of SK&F 96365.
- In contrast, SK&F 96365 did not elevate $[Ca^{2+}]_i$ levels in human eosinophils, but still concentration-dependently inhibited $[Ca^{2+}]_i$ elevation to the subsequent added agonist.
- The selective PKC inhibitor, Ro 31-8220/002, dose-dependently enhanced the $[Ca^{2+}]_i$ elevations to added agonist in guinea pig eosinophils.
- The specific tyrosine kinase inhibitors genistein and herbimycin A, failed to have an effect on either guinea pig eosinophil or unmatured Eol-1 $[Ca^{2+}]_i$ responses to added agonist.
- The specific PI 3-kinase inhibitor, wortmannin, had no effect on $[Ca^{2+}]_i$ elevations in human eosinophils elicited by the chemokine RANTES.



6 Tyrosine Phosphorylation in Eosinophils

6.1 Western blotting experiments

To ascertain whether tyrosine phosphorylation was occurring in either guinea pig eosinophils, human eosinophils or the eosinophilic cell lines Eol-1/ Eol-3, the technique of Western blotting was employed. This method has been successfully used on rabbit platelets (210) and T-lymphocytes (211).

6.1.1 Tyrosine phosphorylation in guinea pig eosinophils

Unstimulated and eosinophils stimulated with C5a and PAF showed extensive tyrosine phosphorylation with the antiphosphotyrosine specific murine monoclonal antibody PY20. To assess very rapid activation, tyrosine phosphorylation was measured at 2sec, 5sec, 10sec, 20sec, and 30sec in a stirred cell system. Despite the enormous amount of phosphorylation, there appeared to be very little up-regulation or down-regulation of the signal (see figure 6.1). Any changes that did occur were inconsistent. This was also the case for tyrosine phosphorylations measured for sustained activation, ie. 1min, 5min, 15min, 30min and 60min. However, when tyrosine phosphorylation was measured for sustained activation in laminin-coated tubes, there did appear to be an increase in the signal at around 60-65, 100 and 120kDa in C5a (100nM) and PAF (100nM) stimulated eosinophils (see figure 6.2). In PAF stimulated cells, a decrease in the 120kDa tyrosine phosphorylated band was observed at 45min, but all the phosphorylated bands were lower in this lane. This suggests unequal loading of the protein onto the gel has occurred. However, as can be also seen in figure 6.2, these increases were also detected in unstimulated cells.

Due to the high degree of basal of tyrosine phosphorylation detected, control

experiments were performed to establish the amount of nonspecific binding of the antibody on the membrane.

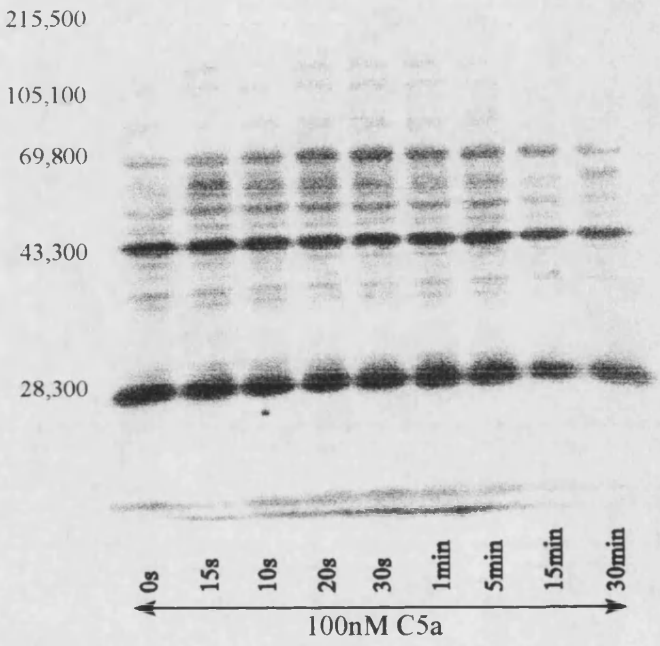


Figure 6.1 Time courses of tyrosine phosphorylation in guinea pig eosinophils. Stirred cells were incubated with C5a (100nM) at 37°C. Samples were run on a 10% SDS-PAGE gel, transferred to nitrocellulose membrane and were detected by Western blotting with PY20 and ECL. Representative of 3 experiments.

Figure 6.2 (see next page) Time courses of tyrosine phosphorylation in guinea pig eosinophils incubated in laminin-coated-tubes. Cells were incubated with vehicle, C5a (100nM) and PAF (100nM) at 37°C. Samples were run on a 10% SDS-PAGE gel, transferred to nitrocellulose membrane and were detected by Western blotting with PY20 and ECL. Representative of 3 experiments.

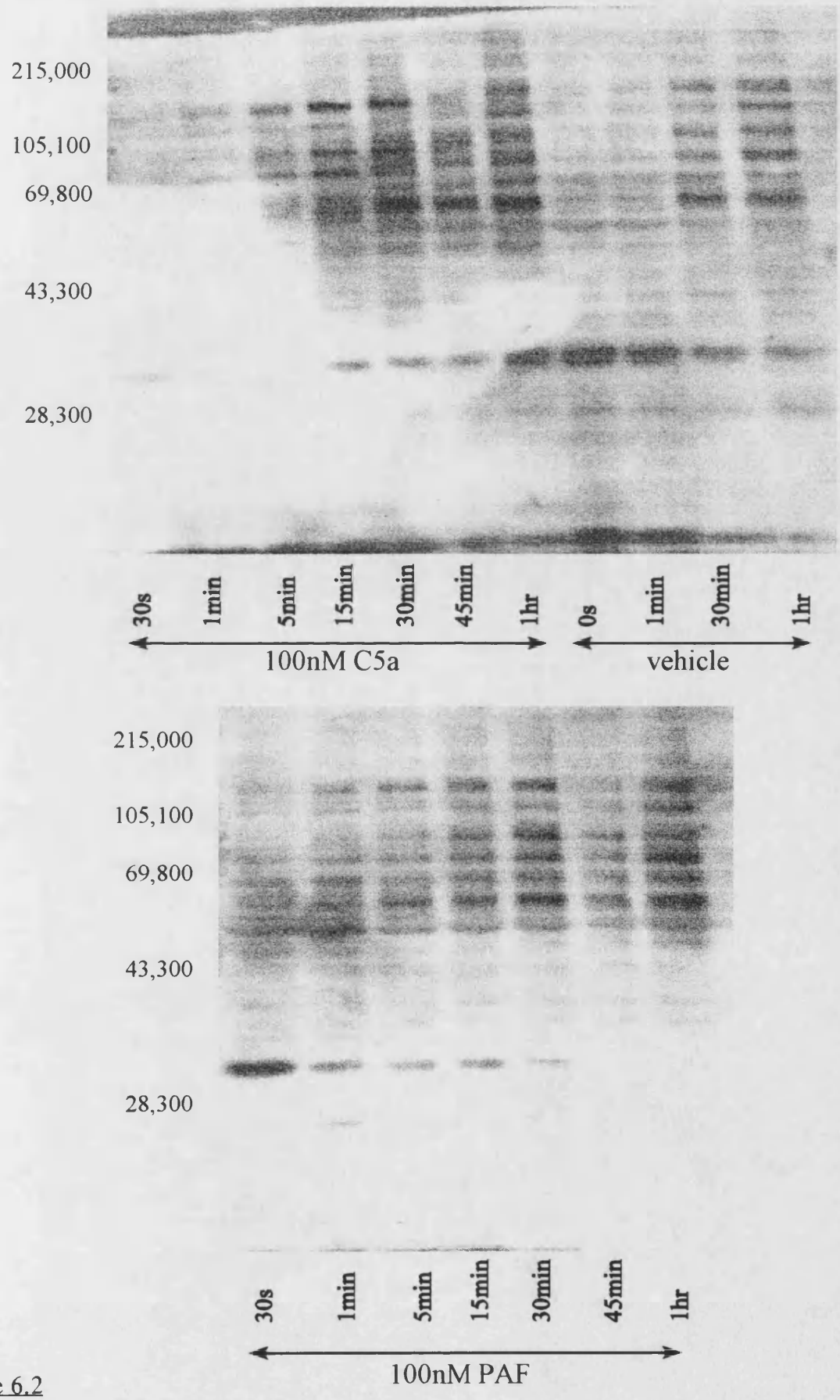

215,000
105,100
69,800
43,300
28,300
30s
1min
5min
15min
30min
45min
1hr
0s
1min
30min
1hr
100nM C5a
vehicle
215,000
105,100
69,800
43,300
28,300
30s
1min
5min
15min
30min
45min
1hr
100nM PAF

Figure 6.2

These included excluding the first antibody (PY20) and substituting the first antibody with whole mouse IgG. Also, the membrane was probed with PY20 in the presence of phosphotyrosine (1mM). These results showed that without the first antibody, no tyrosine signal was detected. The amount of PY20 binding in the presence of phosphotyrosine was slightly lower than PY20 on its own. This suggested that a lot of nonspecific binding had taken place. Extensive binding also occurred with whole mouse IgG. Whole IgG is not an ideal control, as PY20 is an $IgG_{\beta II}$ F_c fragment. Therefore, the nonspecific binding may have been exaggerated in these experiments.

Another antiphosphotyrosine murine monoclonal antibody, 4G10, was also tested. This appeared to be more specific as experiments with 4G10 in the presence of phosphotyrosine showed minimal antibody binding patterns compared to 4G10 on its own. In addition, these patterns were not blocked by phosphoserine or phosphothreonine (see figure 6.3).

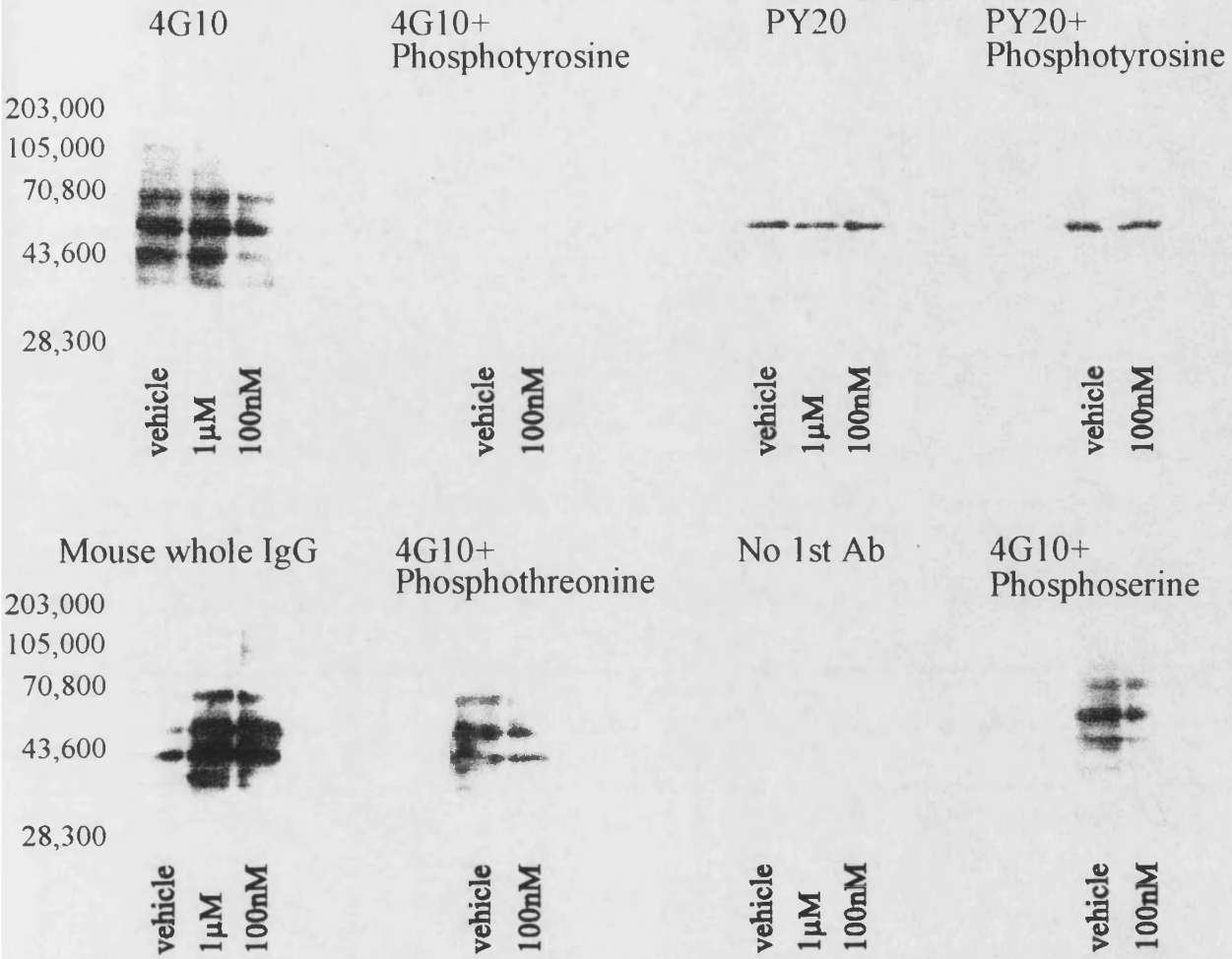


Figure 6.3 Binding of 4G10 and PY20 to blotted eosinophil lysates. Stirred cells were incubated with vehice or C5a (10-100nM) for 30 seconds at 37°C. Samples were run on a 10% SDS-PAGE gel, transferred to nitrocellulose membrane and were detected by Western blotting with PY20 in the presence or absence of phosphotyrosine; 4G10 in the presence or absence of phosphotyrosine, phosphoserine or phosphothreonine; mouse whole IgG, or no first antibody. The blot was developed using ECL. Representative of 2 experiments.

6.1.2 Tyrosine phosphorylation in human eosinophils

Due to the number of eosinophils required to assess tyrosine phosphorylation ($2x10^6$cells/sample), very little work was possible with human eosinophils. Only two tyrosine phosphorylation experiments were performed in laminin-coated microtitre plates. These experiments showed the amount of basal tyrosine phosphorlation detected was less than in guinea pig eosinophil experiments. As figure 6.4 demonstrates, in RANTES (100nM) stimulated cells, the two main bands that appeared to be phosphorylated (detected by 4G10) occurred at 25-30kDa, 60-65kDa and a hint of a band at 110-130kDa. However, there were no significant changes in these bands over time.

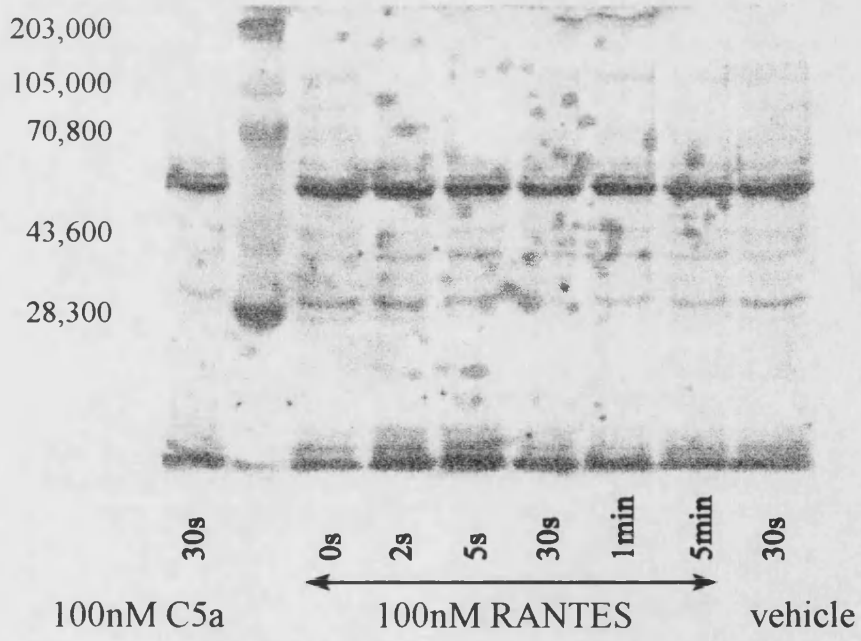


Figure 6.4 Time course of tyrosine phosphorylation in human eosinophils. Cells were incubated with vehicle, C5a (100nM) and RANTES (100nM) fat 37°C. Samples were run on a 10% SDS-PAGE gel, transferred to nitrocellulose membrane and were detected by Western blotting with 4G10 and ECL. Representative of 2 experiments.

6.1.3 Tyrosine phosphorylation in the eosinophilic cell lines, Eol-1 and Eol-3

The advantage of the eosinophilic cell lines was that the experiments were not limited by the number of cells available. The first set of experiments were carried out on unmatured cells. These showed a marked difference in their phosphorylation patterns between the different cell lines, with Eol-1s having a greater extent of tyrosine phosphorylation than Eol-3s. The cell lines were stimulated with C5a (100nM), PAF (100nM), MCP-1 (100nM) and RANTES (100-300nM). None of the phosphorylation patterns (probed with PY20), changed with respect to time (see figures 6.5A & 6.6A for representative tyrosine patterns). The phosphorylation patterns did not change between experiments carried out on stirred cells or cells in laminin-coated tubes.

Control experiments did indicate that no nonspecific binding of the proteins occurred with the antibody PY20, as the binding was completely inhibited by 1mM phosphotyrosine (blank blot not shown).

Figure 6.5 (see next page) Time courses of tyrosine phophorylation in the eosinophilic cell line Eol-1. A) Unmatured Eol-1s were incubated with vehicle or RANTES (100nM) at 37°C. B) Eol-1s matured with butyric acid (0.5mM) were incubated with vehicle or RANTES (100nM) at 37°C. Samples were run on a 7-17% gradient SDS-PAGE gel, transferred to nitrocellulose membrane and were detected by Western blotting with PY20 and ECL. Representative of 2 experiments.

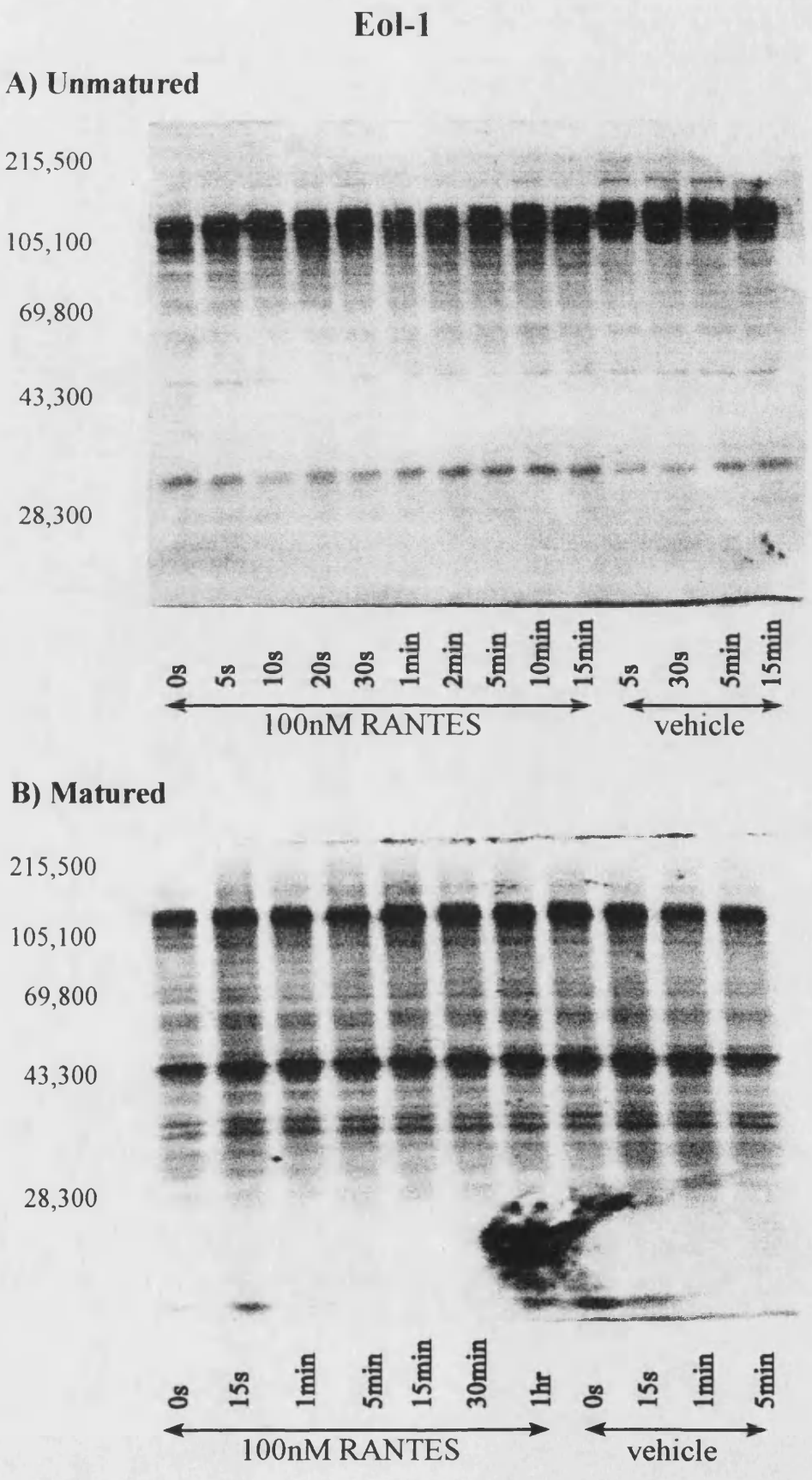

Eol-1
A) Unmatured
215,500
105,100
69,800
43,300
28,300
0s
5s
10s
20s
30s
1min
2min
5min
10min
15min
5s
30s
5min
15min
100nM RANTES
vehicle
B) Matured
215,500
105,100
69,800
43,300
28,300
0s
15s
1min
5min
15min
30min
1hr
0s
15s
1min
5min
100nM RANTES
vehicle

Figure 6.5

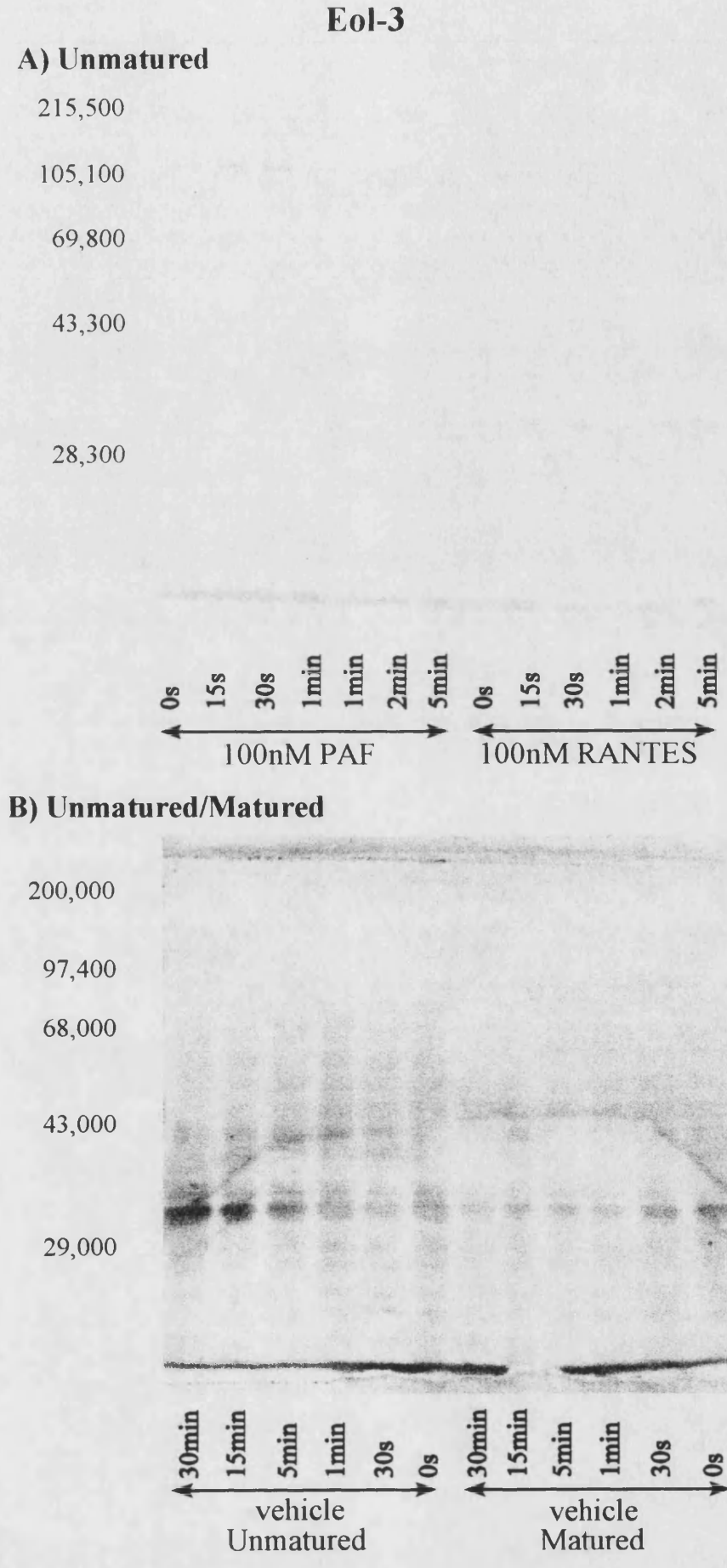

Eol-3
A) Unmatured
215,500
105,100
69,800
43,300
28,300
0s
15s
30s
1min
1min
2min
5min
0s
15s
30s
1min
2min
5min
100nM PAF
100nM RANTES
B) Unmatured/Matured
200,000
97,400
68,000
43,000
29,000
30min
15min
5min
1min
30s
0s
30min
15min
5min
1min
30s
0s
vehicle
Unmatured
vehicle
Matured

Figure 6.6

Upon maturation with butyric acid (0.5mM), there appeared to be some up-regulation of a protein phosphorylated at 44kDa and a down regulation of a protein phosphorylated at 110kDa in the Eol-1s, but no change in the degree of tyrosine phosphorylation occurred in the Eol-3s (see figures 6.5B and 6.6B for representative tyrosine phosphorylation patterns).

6.2 Effect of the tyrosine kinase inhibitors, genistein, herbimycin A and tyrphostin A-47 on tyrosine phosphorylation in guinea pig eosinophils and unmatured Eol-1s

Due to the inability to detect any change in the tyrosine phosphorylation patterns upon agonist stimulation, experiments were performed in the presence of various specific TK inhibitors to see if any changes in the phoshorylation patterns could be invoked.

Guinea pig eosinophils were incubated with herbimycin A (5μM), genistein (300μM), tyrphostin A47 (100μM) and vehicle (0.1% DMSO) for one hour prior to stimulation with C5a (100nM) or HBSS, in laminin-coated eppendorfs.

Figure 6.6 (see previous page) Time courses of tyrosine phophorylation in the eosinophilic cell line Eol-3. A) Unmatured Eol-3s were incubated with PAF (100nM) or RANTES at 37°C. B) Eol-3s either unmatured of matured with butyric acid (0.5mM) were incubated with vehicle at 37°C. Samples were run on a 7-17% gradient SDS-PAGE gel, transferred to nitrocellulose membrane and were detected by Western blotting with PY20 and ECL. Representative of 2 experiments.

As figure 6.7 demonstrates, though C5a treated eosinophils had a slightly increased tyrosine phosphorylation compared with HBSS treated cells, no inhibition of phosphorylation could be achieved in either C5a or HBSS stimulated cells with any of the TK inhibitors. The apparent increase in phosphorylation detected in the tyrphostin experiment was due to a longer exposure of the membrane to the film.

Unmatured Eol-1s, which have extensive tyrosine phosphorylation that does not change upon agonist stimulation, were incubated with the TK inhibitors herbimycin A (875nM), genistein (30μM) and vehicle (0.01% DMSO) overnight. The cells in suspension were then stimulated with MCP-1 (1nM), RANTES (30nM) or HBSS. Though there was no difference between the HBSS treated cells and the agonist-stimulated cells, there was a profound inhibition of the phosphorylation patterns by the TK inhibitors herbimycin A and to a lesser extent genistein (figure 6.8). The viability of these Eol-1s had been reduced by the overnight incubation with TK inhibitors to around 73% in the DMSO and herbimycin A treated Eol-1s, and 40% in the genistein treated Eol-1s. However, the cell concentration was adjusted before the start of the experiment to give equal numbers of viable cells in each sample.

Figure 6.7 (see next page) Time courses of tyrosine phosphorylation in guinea pig eosinophils. Cells were pretreated with vehicle (DMSO 0.1%), herbimycin A (5μM), genistein (300μM) or tyrphostin A-47 (100μM) for 1 hour at 37°C and then stimulated with C5a (100nM) at 37°C. Samples were run on 10% SDS-PAGE gels, transferred to nitrocellulose membranes and were detected by Western blotting with PY20 and ECL. Representative of 2 experiments.

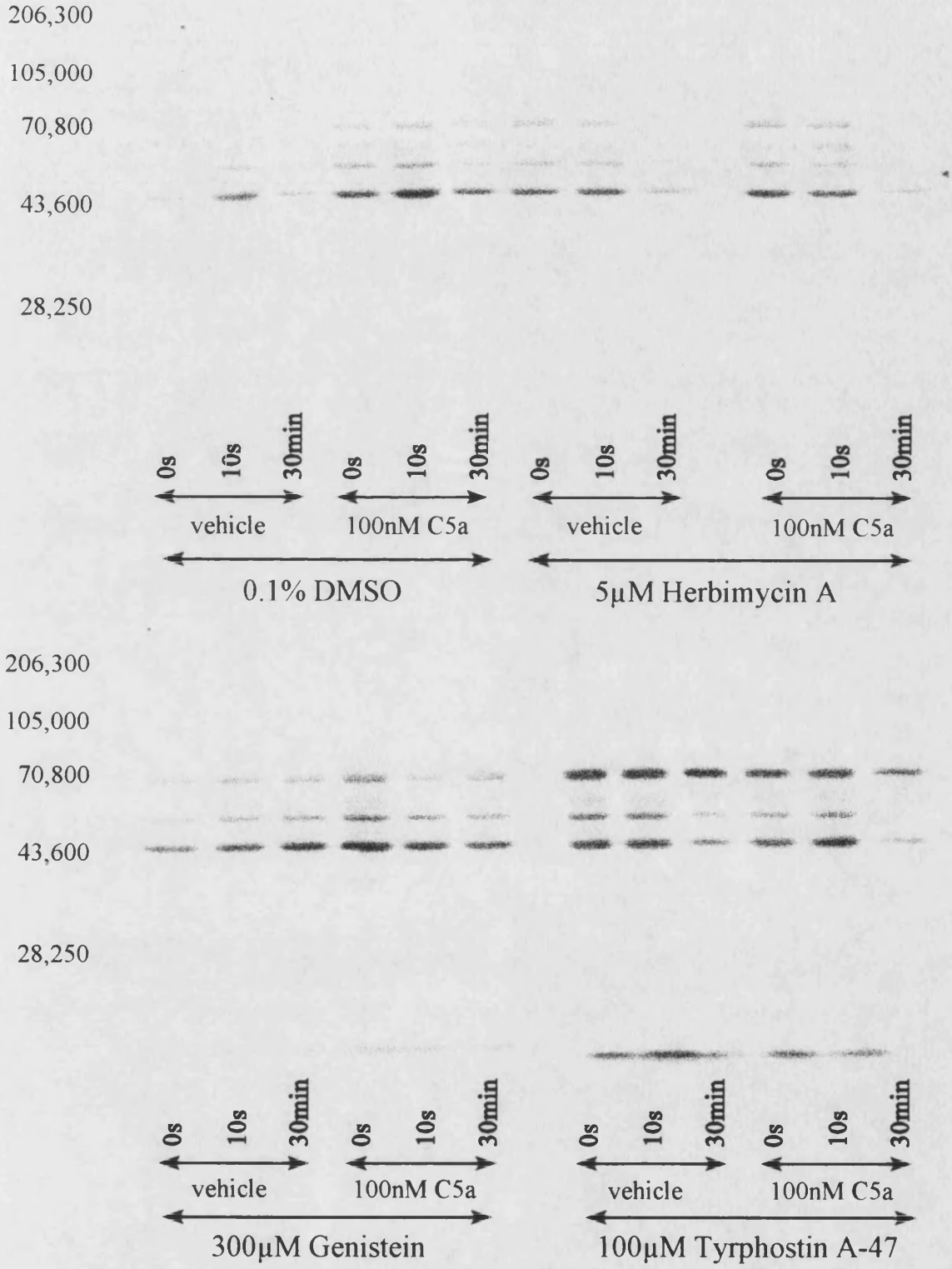

206,300
105,000
70,800
43,600
28,250
0s
10s
30min
vehicle
100nM C5a
0.1% DMSO
5µM Herbimycin A
300µM Genistein
100µM Tyrphostin A-47

Figure 6.7

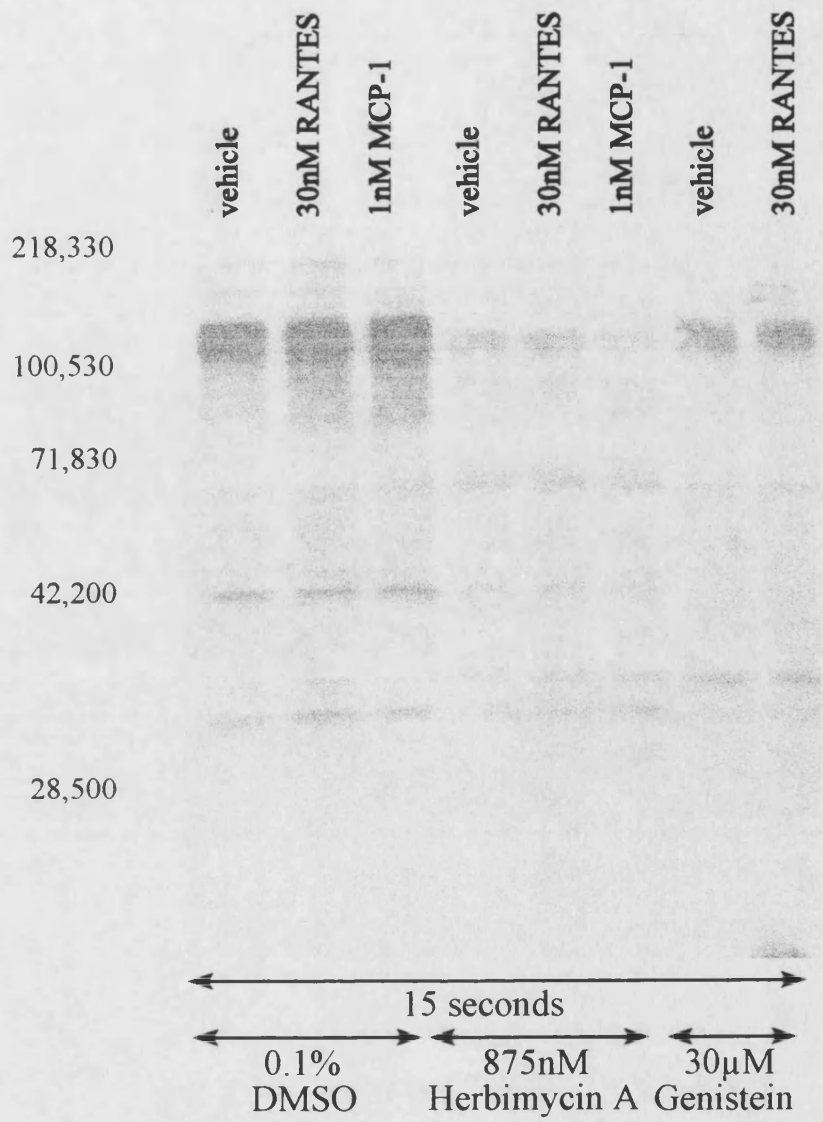
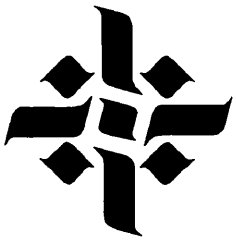


Figure 6.8 Effect of tyrosine kinase inhibitors on tyrosine phosphorylation in Eol-1s. Cells were pretreated with vehicle (DMSO 0.1%), herbimycin A (875nM) or genistein (30μM) overnight at 37°C and then stimulated with either RANTES (30nM) or MCP-1 (1nM). Samples were run on a 7-17% gradient SDS-PAGE gel, transferred to nitrocellulaise membrane and were detected by Western blotting with PY20 and ECL. Figure shows the result from a single experiment.

6.3 Summary of the tyrosine phosphorylation experiments

- Guinea pig eosinophils, human eosinophils and the eosinophilic cell line Eol-1, have extensive protein-tyrosine phosphorylation in their unstimulated and stimulated states.
- The eosinophilic cell line Eol-3 has a much lower tyrosine phosphorylation content.
- An up-regulation of three bands (60-65, 100 & 120 kDa) in guinea pig eosinophils could be induced when unstimulated and stimulated cells were incubated in laminin-coated tubes.
- No up- or down-regulation of the TK patterns could be achieved in either human eosinophils and Eol-1s under any conditions, or in guinea pig eosinophils in suspension to any agonist tested.
- Maturation of the eosinophilic cell lines Eol-1 with butyric acid induced an up-regulation of a 42kDa band and a down-regulation of a 110kDa band. However, maturation of Eol-3s had no effect on phosphorylation patterns.
- The specific inhibitors genistein, herbimycin A and tyrphostin A-47 had no effect on guinea pig eosinophil tyrosine phosphorylation
- Herbimycin A and genistein greatly reduced the amount of tyrosine phosphorylation in unmatured Eol-1s.



7 Discussion

7.1 Initial aims of this project

At the outset of this project, the importance of the eosinophil as a proinflammatory cell in asthma and various respiratory diseases had been well established from clinical studies, but the mechanisms of eosinophil activation were poorly characterised. The purpose of this study was to ascertain what caused eosinophil activation and induce the release of toxic oxygen metabolites. Once particular agents had been identified, these were used to deduce various signal transduction mechanisms that occur during activation. Specific therapeutic agents and subsequent intervention of these mechanisms during eosinophil activation could be useful in preventing these respiratory disorders from occurring.

7.2 Eosinophil H_2O_2 production

Results presented in this thesis show guinea pig eosinophils can be stimulated *in vitro* to produce significant amounts of H_2O_2 with the soluble stimuli C5a and LTB_4. PAF was a weaker agent in eliciting H_2O_2 production at all doses tested. Previously eosinophils had been shown to produce H_2O_2 but substantial release was elicited by the phorbol ester PMA. Particulate stimuli were much less effective than PMA (212). In studies that measured O_2^- production rather that H_2O_2 released, eosinophils have been shown to be capable of eliciting O_2^- in response to various other stimuli. In guinea pig eosinophils this includes LTB_4 (213), digitonin, NaF, A23187, and weakly by PAF and opsonised zymosan (75,107,189).

The advent of the magnetic assisted cell sorter (MACS) in conjunction with a CD16 antibody has made the purification of peripheral blood eosinophils more feasible

(207). CD16 is a molecule present on neutrophils but not eosinophils and using the column, unstimulated eosinophils are negatively selected. Using this technique eosinophils were successfully isolated, and C5a and PAF were demonstrated to produce H_2O_2 from human eosinophils as well as from guinea pig eosinophils. LTB_4 was not tested on human eosinophils, but it has been shown to induce chemotaxis and IgE binding of human eosinophils (214,214a). However, some functional responses have been reported to be lacking in response to LTB_4 suggesting certain biochemical and functional differences exist between different species (214b). Human eosinophils have been shown to release the cationic proteins EPO and ECP in response to PAF and also release O_2 at much higher agonist concentrations (1-30μM) (215). IgA and IgG have also been shown to cause degranulation (by measuring EDN) and this effect was enhanced by the cytokines GM-CSF and IL-3. In addition, a small amount of degranulation was caused by IL-5 (216).

Various cytokines and chemokines have been identified at sites of inflammation and are thought to have a role in recruitment and cellular function. It was thus important to establish whether these played a role in H_2O_2 production from human eosinophils.

It was demonstrated that various human cytokines, which were inactive when added to guinea pig eosinophils, were able to elicit H_2O_2 from human eosinophils. These included IL-5, MCP-3 and RANTES. Interestingly, all the agonist-induced responses followed a similar time course, with the cells releasing H_2O_2 from agonist stimulation reaching a maximal response by 30 minutes, except IL-5, which didn't elicit a maximal response until one hour. The time courses followed similar patterns

at lower doses, again reaching a plateau by 30 minutes or 60 minutes in the case of IL-5. Similarly, other studies have also shown the ability of agents to release H_2O_2 and O_2^- from human eosinophils. Again, PMA has been shown to be very potent (212).

For reasons documented in the introduction, the scopletin based assay to measure H_2O_2 was used. Haynes and Fletcher (217) pointed out that the most accurate way of measuring the respiratory burst is by using the SOD-inhibitable reaction as SOD is specific to superoxide. However care must be taken as H_2O_2 formed by the dismutation can oxidise the reduced ferricytochrome *c* resulting in an underestimation of O_2^-. Using the SOD-inhibitable method, O_2^- production was successfully measured, repeating the same order of potency that was achieved in the H_2O_2 experiments. However, the window of detection was much smaller than in the H_2O_2 assay.

The disadvantage of the scopoletin-based assay is that the method is sensitive to other hydrogen donors such as serum proteins. 75% inhibition could be obtained by catalase, which breaks down the H_2O_2 produced. The scopoletin based assay required fewer cells than the ferricytchrome *c* assay and was monitored with ease in the fluorescent plate reader.

To ascertain that the H_2O_2 being detected was being produced via the NADPH oxidase complex, various inhibitors were used. Diphenylene iodonium first produced by Collette *et al* (218) has been described to inhibit O_2^- generated by the NADPH oxidase by Cross and Jones in neutrophils (219) and Déme *et al* in the thyroid plasma membrane (220). However, this componud was insoluble at the concentrations required for inclusion in the H_2O_2 assay. Another reported NADPH oxidase inhibitor is HMAP (221), which has been shown to attenuate NF-κB induction (which occurs

upon O_2^- generation) in mesangial cells (208). HMAP proved to be a potent inhibitor of NADPH oxidase causing a 90% reduction in H_2O_2 production with no effect on viability. Whether HMAP had any other non-specific activities was not evaluated in this study.

The properties of O_2^- generation and H_2O_2 production have been investigated in great detail in human neutrophils due to the large numbers that can be obtained. Comparing eosinophil responses to neutrophil responses some differences in function exist. Neutrophils on coated surfaces appeared to exhibit a lag period of between 15-90 minutes after addition of added agonist (196). No lag time was observed at all when either guinea pig or human eosinophils were stimulated with any agonist. In the case of guinea pig eosinophils it was thought that the cells were already primed by the method to elicit them, and thus any stimulus that caused H_2O_2 production immediately activated the respiratory burst. However, the fact that human eosinophils can be obtained from peripheral blood without apparent activation would indicate a closer association between the receptor and the respiratory burst. It has been well established that eosinophils have a greater capacity for O_2^- generation than neutrophils (189,222).

Nathan showed that some agents, TNFα/β, were unable to trigger H_2O_2 release when the cells were in suspension, but able to induce a massive, prolonged secretory response when the cells were plated on different protein surfaces. In conjunction with the lag time, this data suggests that effect may be due to the assembly of microfilaments between the cell and membrane proteins before cellular activation (196). Though only a few suspension experiments were carried out for the protein kinase C studies, no difference was detected in activation time between the two types

of experiments. In addition PAF, which was only a poor activator of guinea pig eosinophils in suspension (data not shown) was also a weak activator of eosinophils on laminin-coated plates.

Certain cytokines have the ability to prime granulocytes to enhance their actions. For example DiPersio *et al* have shown that a one hour incubation with GM-CSF can enhance the release of arachidonic acid from neutrophils in response to A23187, fMLP, PAF and LTB_4 (223) and McColl *et al* show increase in the synthesis of LTB_4 in response to GM-CSF in PAF stimulated neutrophils (224). Similarly, IL-3 and IL-5 have been shown by Takafuji *et al* to enhance LTC_4 synthesis by eosinophils in response to C5a and PAF, which were unable to induce LTC_4 on their own (225). With respect to the oxidative mechanism, Weisbart *et al* have shown human neutrophils and a two hour pretreatment with GM-CSF can enhance the oxidative metabolism in response to fMLP, C5a and LTB_4 but not PMA (226) and Yuo *et al* found that a 10 minute preincubation with GM-CSF and TNF could enhance the IL-8 induced oxidative burst from human neutrophils (227). Interestingly, it has been shown that endotoxin is capable of priming neutrophils for enhanced oxidative responses in response to fMLP (226).

Any agonist that was not able to activate eosinophils *in vitro* could not be significantly enhanced by the suggested priming agents GM-CSF, IL-3 and IL-5, though some increases did occur. Even the response elicited by C5a in guinea pig eosinophils could not really be amplified by priming agents. Again the possibility that guinea pig eosinophils were already primed could be responsible for these negative responses.

When the effects of the cytokines RANTES and IL-5 were tested by themselves on human eosinophils, each gave their respective response. When these agents were added together their responses was additive not synergistic. It was thought that RANTES-stimulated human eosinophil responses, could be enhanced with the cytokine IL-5. The result was the same if IL-5 was added prior to the addition of RANTES, or if they were added at the time. However, an investigation of the priming time was not carried out, hence eosinophils may need 2-3 hours before they are upregulated to have enhanced activity to any added agonist. The experiments when the cytokines were added together were left to run for 2½ hours, so any effect on priming should of been observed.

Experiments were carried out on guinea pig eosinophils that were obtained from BALs from non-challenged and challenged animals rather than eosinophils elicited by horse serum in the peritoneal cavity. This was to establish if functional differences existed depending on the method of production. Very few eosinophils were obtained from non-challenged animals. When comparing eosinophils from challenged animals to non-challenged animals it appears that the cells are just as responsive to C5a, but PAF tended to elicit more H_2O_2 from eosinophils in challenged animals. This suggests the inflammation observed in response to OA-challenge enhances the activity of the cell to respond to certain stimuli. This enhanced response could be due to the eosinophils being primed by virtue of their recruitment to the bronchial lumen.

7.3 Eosinophil adhesion

The protein coating of laminin was selected empirically. Initially a broad range

of coatings were used and these were narrowed down on practical cost and effectiveness to coat the plate. For eosinophils, unlike macrophages, coating the plate was necessary to stop spontaneous H_2O_2 release. Fibrinogen and laminin bind the ligands $\alpha_M\beta_2$ (Mac-1), $\alpha_x\beta_2$ (p150,95) and $\alpha_1\beta_1$ (VLA-1), $\alpha_2\beta_1$ (VLA-2), $\alpha_3\beta_1$ (VLA-3), $\alpha_6\beta_1$ (VLA-6), $\alpha_7\beta 1$ (VLA-7) respectively. Eosinophils are known to possess the β_2-integrins, as these are present on all leukocytes and hence make fibrinogen an effective protein surface for the cells to adhere to. The presence of β_1-integrins on eosinophils had not been established except VLA-4, which binds to VCAM-1 and fibronectin. The function of laminin proved very effective for my experiments. Dri *et al* who have tested various physiological stimuli with eosinophils layered onto a variety of biological surfaces found plastic to give much higher O_2^- readings than the different proteins (fibronectin, laminin, collagen type I & IV and fibrinogen) in response to basal, fMLP, TNFα and PAF (228). In agreement with this study, Dri found that eosinophils on laminin-coated surfaces were relatively inactive when stimulated with these agonists.

A functional laminin receptor, VLA-6, has only been recently described on human eosinophils by Georas *et al* (21). They found the presence of α_4 and α_6, but not α_1, α_2, α_3, or α_5 integrin subunits on human eosinophils. They found that the inhibition of eosinophils adhering to laminin could be caused by the anti-α_6 monoclonal antibody, GoH3, and by the anti-β_1 monoclonal antibody, 33B6. Laminin has also been shown to interact with the integrin $\alpha_6\beta_4$ (229), but Georas found no presence of the β_4 integrin subunit on eosinophils either (21).

Not only did eosinophils appear to adhere to laminin for activation of eosinophil

H_2O_2 release, but the level of adhesion in many instances altered depending on the agonist, suggesting a dynamic relationship between these two cellular functions. Guinea pig eosinophils had increased adhesion when stimulated with C5a, LTB_4, PAF and PMA, and human eosinophil adhesion was increased by the agonists C5a, IL-5, MCP-3 and PMA.

In neutrophils a direct relationship between these two functions appears to exist. Shappell *et al* have demonstrated that inhibiting Mac-1 mediated adherence in these cells reduces H_2O_2 release upon fMLP stimulation (230). Walker *et al* demonstrated that eosinophils after migration across IL-1 stimulated HUVEC monolayers had upregulated CD11b and CD35. This was only partially inducible with PAF or a variety of cytokines and it was postulated that cell-cell interactions rather than soluble factors were responsible for the altered eosinophil marker expression. This upregulated CD11b was related to an increased capacity to generate O_2^- when stimulated by opsonized zymosan (231). Therefore adhesion may only increase H_2O_2 release from eosinophils, when certain adhesion molecules have been expressed.

PAF though a weaker activator of H_2O_2 release from guinea pig eosinophils than C5a and LTB_4, was capable of causing eosinophils to adhere to laminin-coated plates to the same extent as C5a and LTB_4. Kimani *et al* identified this PAF induced adherence to be regulated by the β_2-subunit (232). In the case of PAF, it could mean that it is quite important in recruiting the eosinophils as well as other inflammatory cells, but not sufficient to cause major activation. This would avoid incorrect targeting of the functional response, which would otherwise be disasterous.

IL-5 not only caused significant H_2O_2 release from human eosinophils but

upregulated adhesion. As mentioned earlier, the time course of H_2O_2 release from IL-5 stimulated eosinophils was different from other agonists, where IL-5 caused release of H_2O_2 more slowly, and reaching a maximum at one hour rather than 30 minutes. This could indicate a role of adhesion priming the cell before activation (with some cells possibly primed during purification) or after subsequent activation by IL-5, the IL-5 that is possibly released by the cell (233) could maintain the cellular adhesion/increase activation. Indeed the adhesion experiments were measured at the end of the assay, typically 90 minutes, in all experiments, and earlier or later time points could tell another story. Adhesion has been shown to prime cells for activation for other functional responses. For example Anwar *et al* have shown that eosinophils binding to fibronectin via VLA-4, enhanced the cells ability to release LTC_4 to the calcium ionophore A23187. Interestingly, they found optimal adherence to occur at 60 minutes, which was sustained until the end of the assay (120 minutes) (234).

Only 35-50% of the total number of eosinophils (depending on the agonist) adhered to the plate. Total adhesion might not be expected as a proportion of the cells may be non-adherent once they have been activated and released available H_2O_2. A number of eosinophils would be lost in the washing process after the H_2O_2 assay, though this should be a uniform error across the plate. A method for removing the cells with minimal shear force has been suggested by St. John *et al* (235).

7.4 $[Ca^{2+}]_i$ elevation

The elevation of intracellular free calcium is now well documented in both guinea pig and human eosinophils (44,107,213,236,237). These are in agreement with

data presented in this thesis. However, this study demonstrates that in guinea pig eosinophils these responses occur at physiological doses with both C5a and PAF eliciting calcium release at 10nM and LTB_4 causing a significant response at 1nM and above. With respect to human eosinophils, C5a, RANTES, MIP-1α and MCP-3 also caused significant elevations of intracellular free calcium. This has been also demonstrated by Rot *et al* and Dahinden *et al* (44,236). These groups have also carried out cross-desensitisation experiments between MIP-1α and RANTES. This study also establishes that human eosinophils were desensitised to MCP-3 by RANTES, but not vice versa, indicating the MCP-3 responses are mediated by RANTES. This effect has also been demonstrated by Dahinden (236). MCP-1 receptors have been reported not to exist on eosinophils (44), but I found a significant response elicited by MCP-1 in human eosinophils. The experiments in this study imply that the reaction initiated by MCP-1 acts through a receptor that is also activated by MCP-3, which is distinct to the MCP-3/RANTES receptor. Whether this is via an MCP-1 specific receptor or through a different chemokine receptor has yet to be determined. MCP-1 is not acting via the RANTES specific receptor, due to the activity of RANTES after the MCP-1/MCP-3 response.

The first indications of diversity in signal transduction mechanisms became apparent by these observations. In H_2O_2 experiments PAF and MIP-1α were weak activators of the respiratory burst in guinea pig and human eosinophils respectively, whereas both these agonists were potent inducers of free calcium elevation. Conversely, IL-5 was a potent agonist causing a massive secretion of H_2O_2 from human eosinophils, but unable to cause calcium elevation. The PAF and MIP-1α data is in

concordance with a study by Garland on human neutrophils where a rise in intracellular free calcium occurs prior to O_2^- generation, but this calcium signal is insufficient to elicit the respiratory burst (109). IL-5 probably uses a different mechanism for eosinophil H_2O_2 release. Table 7.1 shows a comprehensive list of all the agonists that activate eosinophils and all the agents that are released from eosinophils. This list has been compiled from this study and current literature.

Table 7.1 Eosinophil receptors expressed and agonists produced

Eosinophil Receptors	Role	Reference
GM-CSF	Cell survival	(7)
IFNγ	Cell survival/delayed action	(238)
IL-1α	Inhibits O_2^- production	(239)
IL-2	Priming	(41)
IL-3	Cell survival	(7)
IL-4	Inhibits IgG receptor expression	(240)
IL-5	Cell survival, Chemotaxis, O_2^- production	(7,241,242)
IL-8	Chemotaxis	(243)
LTB_4	Chemotaxis, O_2^- production, $[Ca^{2+}]_i$	(38,214)
MCP-3	Chemotaxis, O_2^- production, $[Ca^{2+}]_i$	(236,242)
MIP-1α	Chemotaxis,$[Ca^{2+}]_i$	(44)
PAF	Chemotaxis, O_2^- production, $[Ca^{2+}]_i$	(36,107)
PDGF	Degranulation	(246)
RANTES	Chemotaxis, O_2^- production, $[Ca^{2+}]_i$	(44,242)
TNFα/β	Enhances LTC_4 production	(247)
Eosinophil Production	**Action**	**Reference**
GM-CSF	Autocrine loop	(86,88)
HLA DR	Interaction with $CD4^+$ lymphocytes & elicit antigen specific responses	(248)
IL-1α	Acute inflammation	(85)
IL-3	Priming	(86)
IL-5	Priming, autocrine loop?	(249)
IL-6	Acute inflammation?	(89)
IL-8	Priming?	(244,245)
LTC_4	Contraction of smooth muscle, oedema & mucus production	(81,250)
MIP-1α	small chemotaxis	(44,90)
PAF	Chemotaxis of granulocytes	(251)
PGD_2	Mucus production & bronchconstricion	(79)
PGE_2	Inhibition of mast cell degranulation	(76,252)
$TGF\alpha/\beta_1$	Chronic inflammation	(83,84,253)
TNFα	Acute inflammation?	(90,91)
TxB_2	Bronchoconstriction	(79)

7.5 A role for extracellular divalent cations in eosinophil activation?

The calcium response caused by LTB_4 was biphasic, and it is postulated that the first rise in intracellular concentration was mobilization from the intracellular stores and the second phase of the response was due to calcium influx.

This was on the basis that agonist-induced Ca^{2+} influx in human neutrophils was reported by Montero *et al* to be secondary to the emptying of intracellular Ca^{2+} stores (121). They found that release of Ca^{2+} from the internal stores activates the plasma-membrane Ca^{2+} channels by a mechanism involving microsomal cytochrome *P*-450.

Experiments were performed to ascertain whether calcium influx did occur. Removal of extracellular calcium inhibited $[Ca^{2+}]_i$ elevation and suggested a large calcium influx. However, the replacement of calcium with manganese and nickel appeared to have no effect on calcium influx suggesting the eosinophils in the first set of experiments were probably store depleted.

Another tool available for investigating calcium influx is the proposed ROCC blocker SK&F 96365. In guinea pig eosinophils, SK&F 96365 was found to have an agonistic effect, though it did subsequently dose-dependently inhibit a rise of $[Ca^{2+}]_i$ in cells stimulated by C5a and PAF. In contrast to these experiments, SK&F 96365 caused no elevations of intracellular free calcium in human eosinophils (except at 100μM), but still caused a dose-dependent inhibition of subsequent calcium elevations in cells stimulated by C5a, PAF and RANTES. Grix *et al* have shown that eosinophil calcium responses do have a calcium influx component using the surrogate calcium ion, manganese (254).

The specificity of SK&F 96365 for ROCCs has been questioned. Merritt *et al*

have used this compound in conjunction with human platelets, neutrophils and endothelial cells and SK&F 96365 has blocked receptor-mediated calcium entry over internal release. SK&F 96365 also blocked manganese influx into platelets and neutrophils. However, pituitary cells and rabbit ear-artery smooth-muscle cells held under voltage-clamp were also inhibited by SK&F 96365 suggesting an effect on VOCCs, though no VOCCs have been identified on eosinophils. Interestingly, the ATP-gated Ca^{2+}-permeable channels of the rabbit ear-artery smooth muscle cells were unaffected by SK&F 96365 suggesting that it may be able to discriminate between some type of ROCCs channels (117,255).

Mason *et al* used SK&F 96365 and other imidazoles to investigate the role cytochrome *P*-450 has in regulating the plasma membrane Ca^{2+} permeability, by the release of Ca^{2+} from the Ins (1,4,5)P_3-sensitive pool. In addition they found SK&F 96365 caused a release of Ca^{2+} from thapsigargin-sensitive intracellular stores, consistent with inhibition of sarcoplasmic reticulum Ca^{2+}-ATPase. They concluded that SK&F 96365 inhibited cytochrome *P*-450-independent filling of intracellular pools, and store-regulated Ca^{2+} entry (120). Perhaps this is the action of SK&F 96365 on guinea pig eosinophils, or alternatively the tertiary structure of SK&F 96365 may be so arranged that it binds to eosinophil membrane receptors in general. The subsequent inhibition of elevation in free calcium may be a desensitisation effect. When PAF was added prior to SK&F 96365, both agents were able elevate intracellular free calcium concentrations. This suggests that the actions for SK&F 96365 if via receptors, were not specific to one type.

The mechanism of direct non-specific action via agonist receptors would explain

the release of H_2O_2 from guinea pig eosinophils, as SK&F 96365 was a potent stimulator of the respiratory burst.

As with the human calcium experiments the effect of SK&F 96365 was quite different on human eosinophils when looking at H_2O_2 release. It appears from preliminary experiments that SK&F 96365 was able to inhibit H_2O_2 production. Perhaps there is a calcium influx component that is necessary for activation or formation of the NADPH oxidase complex, especially if a role for PKC is implicated in the signalling pathway, which requires calcium for activation. These particular actions of SK&F 96365 have not yet been reported in the literature.

The role of extracellular divalent cations on H_2O_2 release from guinea pig eosinophils were investigated. Experiments showed that the removal of calcium inhibited the amount of H_2O_2 produced from eosinophils by approximately 50% in each of the agonists tested, whereas removal of magnesium as well as calcium caused total inhibition. Jones *et al* and Aoyagi *et al* (94,115) have already implied that magnesium is important in the NADPH oxidase complex in neutrophils, possibly through a G-protein and this work demonstrates that eosinophils are also dependent on extracellular magnesium.

This work is also in agreement with Yamashita & Someya who showed both calcium and magnesium were necessary for maximal O_2^- production from guinea pig eosinophils stimulated with A23187. However, they reported magnesium inhibited O_2^- generation at low extracellular calcium concentrations (<0.2mM) (256). This may be due to inhibition of ion influx from the medium into the cell, which may be required for activation of the NADPH oxidase complex.

Ca^{2+} and Mg^{2+} are clearly required for the maintenance of the generation of H_2O_2 from guinea pig eosinophils as addition of EDTA at the beginning or during the middle of an experiment stops the production of H_2O_2 immediately. The addition of nickel, which blocks the ion channels, either at the beginning of the experiment or during the experiment, took about two minutes before the production of H_2O_2 ceased. This difference in action may be purely dynamic. With the addition of a high concentration of EDTA not only is the extracellular calcium chelated, but almost immediately the cell would be store depleted, and probably cause the disassembly of the NADPH oxidase complex and/or other cellular components. However, nickel, as well as steric hindrance, may slowly cause an imbalance of ionic conditions (as extracellular calcium was still present), due to the blocked channels. With the calcium channels blocked a negative feed back system might cause calcium release from the intracellular pools to stop, when the $[Ca^{2+}]_i$ exceeded critical levels. This could subsequently lead to cellular deactivation. Azula *et al* have shown that nickel does not only block Ca^{2+} influx but dose-dependently inhibits platelet aggregation, mobilization and PLC activation induced by thrombin, PMA and A23187 (257).

Adhesion experiments were also disrupted by the removal of the divalent cations. As mentioned in the introduction, the integrins have potential divalent cation binding sites. The removal of Ca^{2+} appeared to have little effect on guinea pig adhesion, if anything it consistently enhanced the adhesion of guinea pig eosinophils to laminin-coated plates. The removal of both Ca^{2+} and Mg^{2+} significantly inhibited the adhesion in response to C5a and PMA, suggesting that Mg^{2+} is the important divalent cation in eosinophil adhesion. Dobrina *et al* showed that the adhesive

mechanisms of eosinophils to be optimally active in the presence of both Ca^{2+} and Mg^{2+} and this was reduced by removal of either cation. It appears that VCAM-1 and VLA-4 are only active when both Ca^{2+} and Mg^{2+} are present. Furthermore the CD11/CD18 integrin was inactive in calcium medium only (258). This is in agreement with this study where the removal of both cations reduces the adhesion of eosinophils when stimulated with added agonist.

Lundgen-Akerlund *et al* have indicated that adhesion was dependent on Mg^{2+}, but not Ca^{2+}, and the addition of manganese lead to enhanced neutrophil adhesion *in vitro* to protein coated surfaces (114). Manganese competes with divalent cations and binds to the integrin with higher affinity, which is thought to induce a conformational change (113).

This study also investigated the role of Ni^{2+} and it was found that this ion markedly increases guinea pig eosinophil adhesion in unstimulated and stimulated cells. Ni^{2+} blocks the calcium influx channels. From this information and experiments with EGTA, extracellular Ca^{2+} does not appear to be essential for adhesion, but may have a role in 'anti-adhesion' after cellular activation.

When the ROCC blocker SK&F 96365 was tested for its role in adhesion, on unstimulated eosinophils, it had little or no effect. This suggested its action on H_2O_2 release was direct, with no consequences on the cellular adhesion mechanisms. However, when added in conjunction with added agonist, SK&F 96365 reduced the eosinophil adhesion below basal levels. When SK&F 96365 was added in conjunction with added agonist in H_2O_2 experiments, the eosinophil was enhanced to release more H_2O_2, so again this loss of adhesion may be due to eosinophils becoming redundant

after activation.

7.6 Action of PKC

Various cellular functions are believed to act via activation of PKC. For example, the chemotactic factor PAF binds with the cell surface receptor and in turn activates PLC. PLC then activates phosphatidyl inositol turnover and the resulting DAG activates PKC (75). PMA, a DAG substitute, bypasses this pathway to act directly on PKC. PMA-induced eosinophil H_2O_2 production was significantly inhibited using the selective PKC inhibitor Ro 31-8220/002 at concentrations of 300nM and above. The responses to soluble agonists were also dose-dependently inhibited by the PKC inhibitor by approximately 80% and 90% in guinea pig and human eosinophils respectively. This strongly indicates that activation of the NADPH oxidase complex occurs via a PKC-mediated phosphorylation of a component of the oxidase, which has been previously demonstrated in neutrophils by Cross and Jones (219).

Shute *et al* (75), have already shown that PKC is involved with O_2^- generation with the inhibitor trifluoperazine. They demonstrated a more potent inhibition of PKC than shown here, but Ro 31-8220/002 is believed to be a more specific PKC inhibitor. The IC_{50} of Ro 31-8220/002 for inhibition of platelet aggregation was shown to be $0.7\mu M$ by Murphy and Westwick (134), which is comparable to the effective concentrations used here.

As stated in the introduction, there are various PKC isozymes and these are poorly characterised in eosinophils. In neutrophils the main isozyme was found to be PKC-β, with some PKC-α and PKC-ζ (132). Several studies have identified a

correlation between PKC activation and the respiratory burst (130,131).

A study by Bates *et al* using western blotting with isozyme-specific mAbs, has detected that PKC-β is present in human eosinophils, but not PKC-α or PKC-γ. They also correlated the activity of PKC to normodense and hypodense eosinophils and found the activity of PKC is increased in hypodense eosinophils. LTC_4 and O_2^- generation are also increased in hypodense eosinophils, suggesting PKC activity is related to cell density in blood-derived cells and cellular function (260). However, Ali *et al* have demonstrated that in the eosinophilic-like cell line, HL-60, LTC_4 synthesis is specifically inhibited by activation of PKC. This inhibition was restored using staurosporine and bisindolylmaleimide (261).

Bisindolylmaleimide is reported to be a potent and selective inhibitor of PKC (262). A recent investigation by Wenzel-Seifert *et al* using a bisindolylmaleimide, GF109203X, which inhibits both c- and n-PKC isozymes and an indocarbazole, Go6976, which inhibits c-PKC isozymes only, have shown that n-PKC isozymes rather than c-PKC isozymes may regulate neutrophil functions including O_2^- generation (263). This would suggest that other isozymes yet to be identified may be present in both neutrophils and eosinophils. The bisindolylmaleimide, Ro 31-8220/002 used in my studies, inhibits preferentially PKC-α over PKC-β, -γ and -ε (264), indicating that a role does exists for c-PKC isozymes in H_2O_2 production in eosinophils. More specific inhibitors of PKC isozymes need to be designed if an individual PKC isozyme is to be associated with a particular cellular function.

A role of PKC in adhesion of guinea pig eosinophils was also apparent, by virtue of the ability of Ro 31-8220/002 to dose-dependently inhibit eosinophil adhesion

in C5a, LTB_4 and PMA stimulated cells. These inhibitions were significant in C5a and PMA stimulated cells. Basal adhesion was unaffected by Ro 31-8220/002. Previous experiments have suggested a dissociation of mechanisms exist between eosinophil H_2O_2 production and cellular adhesion, but this PKC data could indicate an important dependency of adhesion before subsequent activation. To investigate this hypothesis, the PKC inhibitor was used in the stirred cell system, thus preventing adhesion (though not aggregation), to monitor H_2O_2 production from guinea pig eosinophils. In both C5a and PMA stimulated cells, Ro 31-8220/002 prevented the release of H_2O_2, indicating that *in vitro*, though PKC is involved in both adhesion and the respiratory burst, the respiratory burst is not dependent on the eosinophils becoming adherent. However, the adhesion step could enhance the capability of the cells to produce H_2O_2.

Merrill *et al* have identified two pathways of CD11b/CD18-mediated neutrophil aggregation. One pathway, PMA stimulated neutrophils, appears to be sensitive to PKC inhibition (though the PKC inhibitor used was staurosporine). However the other pathway, in fMLP stimulated neutrophils, aggregation is enhanced by staurosporine. It is thought that the PKC acts via the CD18 β-chain as a predominance of phosphoserine residues in CD18 β-chains have been identified on PMA-treated monocytes (265). Sullivan *et al* have also investigated the role of PKC in upregulation of Mac-1 using the selective PKC inhibitor Ro 31-8425. In agreement with Merrill *et al*, they found that phorbol stimulated adhesion could be inhibited by Ro 31-8425 suggesting a PKC mechanism, but adhesion could not be down-regulated when more physiological agonists such as C5a were used to stimulate neutrophils (266). Assuming the β-integrins are similar in eosinophils, the inhibitions observed in this study could

point to a PKC binding site present on or associated with VLA-4, which is specific for eosinophil adhesion.

In some H_2O_2 production and adhesion experiments, there appeared to be an increase in functional response to agonist stimulated cells incubated with the highest inhibitor dose used (3μM). This could be due to the precipitation of the drug or an activation itself by the high concentration. Indeed a similar effect has been documented by Perkins *et al* stating that Ro 31-8220/002 (10μM), though causing a slower rate of H_2O_2 release, actually increased the final amount of H_2O_2 produced. They have speculated that PKC has an inhibitory action on phosphoinositidase C (267).

The significant potentiation of $[Ca^{2+}]_i$ elevation in guinea pig eosinophils by Ro 31-8220/002 suggests that there is an association with PKC and free calcium elevation. As already indicated, calcium elevation in eosinophils, though predominantly mobilization, may also be subject to an influx component. The increase in the second peak in the LTB_4-induced response could suggest that PKC has a negative feedback control on Ca^{2+} influx. However, very little influx was identified in eosinophil experiments, suggesting the PKC negative feedback may act on either DAG or the intracellular calcium stores. This also underlines the principle that a rise in $[Ca^{2+}]_i$ is not sufficient to activate the NADPH oxidase complex and thus cause H_2O_2 release.

Grinstein and Furuya have found in neutrophils, cell activation is not just associated with PKC. Using various inhibitors, receptor-mediated stimulation persisted implying a role for other kinases (268). Souness *et al* have characterised phosphodiesterase activity in guinea pig eosinophils. They found the predominant form of cAMP phosphodiesterase (Type IV) to be located in the membranes, and inhibition

of O_2^- could be achieved with Ro 20-1724, rolipram and denbufylline (269). cAMP is linked to cAMP-dependent protein kinases, which catalyse the transfer of the terminal phosphate group from ATP to specific serine or threonine residues of selected target proteins. This covalent phosphorylation regulates the activity of these proteins (100).

7.7 Tyrosine kinase interaction

Grinstein and Furuya implicated tyrosine phosphorylation with MAP kinase as a result of fMLP chemotactic stimulation in neutrophils. Rapid phosphorylation was evident within 15 seconds. The activation of tyrosine kinase also mimicked the time course and profile of the respiratory burst to fMLP in neutrophils. In their investigation they also noted threonine residues could also have been phosphorylated (270). Dusi *et al* have shown that tyrosine kinase phosphorylation of MAP kinase and an unidentified protein of 75kDa is involved in NADPH oxidase activation, which is coupled to Ca^{2+}-dependent and Ca^{2+}-independent mechanisms in human neutrophils stimulated with fMLP and concanavalin A respectively. However, concanavalin A still requires a Ca^{2+}-dependent mechanism to activate the NADPH oxidase complex (271).

The fact that the PKC inhibitor could not inhibit H_2O_2 production or adhesion by more than 80-90% indicated that other signalling mechanisms may be involved, which may be redundant during normal cellular activation. Several experiments were undertaken to ascertain whether tyrosine kinase signalling was involved in eosinophil activation. These experiments involved using specific tyrosine kinase inhibitors in the functional assays and looking at the phosphorylation patterns.

Using the tyrosine kinase inhibitors, H_2O_2 production from C5a-stimulated

guinea pig eosinophils could be inhibited by a small degree with herbimycin A and genistein, whereas only herbimycin A appeared to inhibit PMA-stimulated cells. The effect of herbimycin A was always consistent, though significance was not achieved. The role of tyrosine phosphorylation in the respiratory burst is still poorly understood. Grinstein and Furuya demonstrated that the accumulation of phosphotyrosine and activation of the NADPH oxidase in neutrophils had similar time courses and concentration dependency when stimulated with $GTP_{\gamma}S$ suggesting a relationship between these mechanisms (272). However, there is a certain dissociation between these two events, as activation of PKC though stimulating oxygen consumption, appears to inhibit tyrosine kinase(s), implying PKC may be part of a negative feedback mechanism intended to control or terminate the responses generated by tyrosine phosphorylation (272).

A dependent/independent role of tyrosine kinase has also been established by Kusunoki *et al.* They showed that O_2^- generation from neutrophils elicited by fMLP and soluble aggregated IgG were inhibited by the tyrosine inhibitors erbstatin and genistein, but O_2^- production by surface bound IgG was scarcely inhibited by either inhibitor. However, immunoblotting studies revealed specific tyrosine phosphorylation of a 40kDa protein by all three agents, and this could be inhibited by the tyrosine kinase inhibitors (273). Azuma *et al* have echoed this work finding a 42kDa protein that is tyrosine phosphorylated (presumably the same protein as the 40kDa protein detected by Kusunoki (273)). They postulated that the respiratory burst elicited by fMLP in human neutrophils is activated by pathways other than PKC and an increase in $[Ca^{2+}]_i$. The tyrosine phosphorylation is induced by PKC-dependent and

independent mechanisms according to the stimuli, indicating that tyrosine phosphorylation is not necessarily related to activation of the respiratory burst (274). Uings *et al* have shown using specific inhibitors that tyrosine kinase is thought to be involved in receptor coupling to PLD but not to the phosphatidyl inositol-4,5 bisphosphate-specific PLC, which in turn activates PKC (275).

Experiments by Fialkow *et al* have demonstrated that tyrosine phosphorylation is enhanced by exogenous oxidants such as diamide and direct stimulation of the GTP-binding proteins. This latter tyrosine phosphorylation was found to be NADPH-dependent as O_2^- generation was inhibited by DPI (an inhibitor of the flavoprotein of the NADPH oxidase (219)). In addition, patients with CGD, which are deficient in the production of reactive oxygen intermediates, demonstrated no such phosphotyrosine accumulation. The various tyrosine phosphorylation bands found to be enhanced were 28, 42-44, 55, 68-70, and 90-110kDa (276).

In contrast to the small reduction of H_2O_2 release detected in this study, the tyrosine kinase inhibitors consistently enhanced the adhesion of guinea pig eosinophils in both vehicle and agonist-stimulated cells. Once again this highlights the divergence of mechanisms between cellular adhesion and the respiratory burst. Tiisala *et al* have also demonstrated this enhanced adhesive effect, where genistein (40μM) upregulated the surface expression of β_2-integrins in the monoblastic THP-1 and the promyelocytic HL-60 cell lines.

Genistein is also thought to modulate the expression of ICAM-1 (277). However, Naccache *et al* have found erbstatin and herbimycin A to inhibit the expression of CD11b and CD18 in human neutrophils, stimulated by the chemotactic

inhibitors fMLP and LTB_4. The anti-CD11b induces the phosphorylation of a 120kDa substrate (278). This could be the same protein detected in the tyrosine phosphorylation experiments reported in this thesis, where C5a and PAF induced the phosphorylation of a 120kDa protein in human eosinophils. This would suggest that after 30 minutes the expression of CD11b may decline with a corresponding increase in tyrosine phosphorylation.

The enhanced adhesion effect that herbimycin A and genistein had in these studies could indicate a different tyrosine phosphorylation pathway distinct from CD11b/CD18, possibly VLA-4 or VLA-6. The effect detected by Tiisala *et al* may be due to the long incubation of the inhibitor (72 hours) with the cells, which are not stimulated with any agonist, suggesting an effect on differentiation/proliferation rather than on function (277).

The tyrosine kinase inhibitors had no effect on the phosphorylation patterns of guinea pig eosinophils. This was surprising considering the extensive phosphorylation that exists in the cells. A more detailed study of the incubation times of the inhibitors is required.

No effect was found of the tyrosine kinase inhibitors on $[Ca^{2+}]_i$ elevation in guinea pig eosinophils. This again may be due to the short incubation times of the eosinophils with the inhibitors. However, even Eol-1s, which were incubated with the inhibitors overnight, failed to show any differences between vehicle and agonist-stimulated cells. This could suggest the action of tyrosine kinase is calcium independent. Nevertheless, Murphy *et al* have shown that genistein (IC_{50} around 100μM) is an inhibitor of PAF-induced $[Ca^{2+}]_i$ elevation in rabbit platelets, affecting

both influx and mobilization. This suggests genistein-sensitive PTKs regulate $[Ca^{2+}]_i$ elevation either directly or indirectly (210).

The results obtained from western blotting experiments were disappointing. Guinea pig eosinophils appear to be heavily phosphorylated before any stimulation, with some strong bands at 25kDa, 42kDa and 68kDa. No change was observed upon stimulation in the stirred-cell system. Under the same conditions as the H_2O_2 assay, there was an upregulation of some bands over time. These were observed in both vehicle- and agonist-stimulated cells. The bands were 60-65kDa, 100kDa and 120kDa. The strong phosphorylation present on the blot is most likely due to mechanisms activated upon transmigration, as the guinea pig eosinophils are elicited into the peritoneal cavity rather than obtained from peripheral blood as is the case for human eosinophils.

This hypothesis is reiterated when the blots of human eosinophils are observed. These have fewer PTK phosphorylated proteins than guinea pig eosinophils. The increase in the band in the laminin-coated eppendorfs must be due to some adhesive property or aggregation. When platelets aggregate three distinct proteins are tyrosine phosphorylated, 35-45, 66-90 and 90-150kDa (210). Two of these three bands coincide with patterns observed in guinea pig eosinophils. However, platelet aggregation occurs rapidly and the changes in phosphorylation are observed by 2 seconds, whereas the changes in guinea pig eosinophils are not observed for 5 minutes but are sustained for up to an hour.

The blots obtained from human eosinophil experiments demonstrate phosphorylation of two main bands, one at 30kDa and another, a doublet, at 60kDa.

Any tyrosine phosphorylated bands at low molecular weights are not separated on the 10% gels. Again no changes were observed upon stimulation, suggesting protein-tyrosine kinases are not involved in the NADPH oxidase complex and the subsequent respiratory burst.

Very few studies of tyrosine kinase phosphorylation on eosinophils have been reported. Van der Bruggen *et al* demonstrated that priming of the respiratory burst in human eosinophils with GM-CSF, IL-3 and IL-5 did not cause a change in $[Ca^{2+}]_i$ but did induce tyrosine phosphorylation of several proteins. The 102 and 122 kDa proteins appeared to be Ca^{2+} independent whereas the 66kDa protein appeared to be dependent on Ca^{2+}. They suggested that the 122kDa protein could be the β-chain of the GM-CSF, IL-3 and IL-5 receptor. Erbstatin, an inhibitor of PTK, not only inhibited the cytokine-induced tyrosine phosphorylation, but also inhibited the respiratory burst (279).

Recently IL-5 has been shown to activate cells via a tyrosine phosphorylation pathway in an IL-5-dependent murine early B cell line by Sato *et al* (280) and in human eosinophils by van der Bruggen *et al* (29). Both these have reported that the IL-5 signalling involves JAK2 kinase (a 130kDa tyrosine kinase protein) and van der Bruggen also showed that JAK2 subsequently activates a member of the Stat (signal transducers and activators of transcription) family, Stat1α (29).

The guinea pig and human eosinophil experiments were always limited by cell number and it was hoped that more information could be obtained from the eosinophilic cell lines Eol-1 and Eol-3. The cell lines have very different basal patterns compared to either the guinea pig or human eosinophils. The Eol-1's have a strong

band at 110kDa and a weaker band at 30kDa. Upon maturation with butyric acid there appears to be a change in the phosphorylation. The strong band at 110kDa is less intense, whereas a band at 43kDa becomes upregulated, the band at 30kDa either becomes a doublet, or is down regulated and a different protein near this molecular weight is phosphorylated in the form of a doublet. Again, no change is observed over time with agonist-treated cells. The Eol-3 cell line had a very weak basal phosphorylation compared to the Eol-1s. However, the same bands of 110kDa and 30kDa were present. No changes in phosphorylation were observed upon maturation or stimulation.

The main difference between Eol-1's and Eol-3's was the rate at which the cells proliferated. Eol-1s could grow from $5x10^5$cells/ml to $2x10^6$cells/ml every three/four days, whereas Eol-3s only grew from $5x10^5$cells/ml to $1x10^6$cells/ml every week. A family of proto-oncogenes that are phosphorylated on tyrosine are thought to encode proteins that are involved in the cascade of events by which growth factors stimulate normal cell division (281). This could account for the differences between the Eol cell lines and the change in patterns on Eol-1 maturation.

Eol-1s did show a marked reduction in tyrosine phosphorylation patterns with an overnight incubation with the tyrosine kinase inhibitors genistein and herbimycin A. This is almost certain to be associated with the inhibition of proliferation, as the total number of cells was reduced with each inhibitor tested. This also affected the relative viability.

7.8 PI3-kinase interaction

As stated in the introduction, PI3-kinase is thought to be a signalling molecule in its own right. It can be activated as a result of tyrosine phosphorylation or other kinases such as PKC-ζ. Wortmannin, reported to be a specific inhibitor of PI3-kinase at nanomolar concentrations (183), was found to inhibit agonist-stimulated H_2O_2 release from both guinea pig and human eosinophils, and agonist-stimulated adhesion of guinea pig eosinophils. However, it was unable to inhibit or change the agonist-induced responses in $[Ca^{2+}]_i$ experiments. It is unlikely that these effects are through a tyrosine-phosphorylated pathway due to the lack of inhibition the PTK inhibitors appeared to have in eosinophils and the significant effect of wortmannin.

Wortmannin did not appear to be acting non-specifically as H_2O_2 could still be elicited from eosinophils by a submaximal dose of PMA and no effect of wortmannin was demonstrated on elevation of $[Ca^{2+}]_i$. Only two reports have previously documented the effects of wortmannin on eosinophils. One by Kapp *et al* but the concentration used was 1μM, which could result in non-specific effects and no data was actually presented (282). The second was by Bach *et al* when the actions of wortmannin were not understood (283).

The data presented in this thesis implies that PKC-ζ could be the molecule activated by PI3-kinase as this is not activated by DAG or phorbol esters (282), and wortmannin failed to inhibit H_2O_2 elicited by PMA in human eosinophils. Many reports suggest that wortmannin blocks the activation of PLD (284,285), but wortmannin does not inhibit PMA-induced PLD activity and even enhances the production of phosphatidic acid in some cell types. Therefore PLD is unlikely to be

the molecular target for the inhibitor (183). However, PI3-kinase could be upstream of PLD and this would account for some of the differences reported in the literature.

Wortmannin has also been reported to block the activation of the MAP kinase pathway in guinea pig neutrophils (286) and in L6 skeletal muscle cells (287). MAP kinase is a PTK of 42-44kDa, which is in turn activated by a protein cascade thought to involve first the GTP-binding protein *ras* and then the serine/threonine kinases and the MAP kinase kinase (287). The role of PI3-kinase in this pathway is still poorly understood and if it exists PI3-kinase may be the signalling step between *ras* and *raf*. PI3-kinase has been connected with the respiratory burst in human neutrophils (183,288) and my data suggests this is also true for eosinophils.

The effect of wortmannin on adhesion implies that PI3-kinase also regulates the expression of adhesion integrins. To my knowledge this is the first data reported connecting leukocyte adhesion and PI3-kinase.

7.9 Comparison of functional studies between guinea pig and human eosinophils

At the outset of the project, human eosinophils were difficult to purify and the yield and percentage of purity were low. Guinea pig eosinophils were used as a good source of eosinophils where high numbers could be easily induced. I successfully demonstrated that guinea pig eosinophils upon agonist-stimulation could adhere, produce H_2O_2 and elevate $[Ca^{2+}]_i$. One of the drawbacks of this model was the relatively few number of agonists that could activate the cell. This was especially disappointing with the increasing field of cytokines, which have been implicated in numerous inflammatory responses. With the magnetic assisted cell sorter, human

eosinophils could be successfully purified and this study demonstrated, as suspected, that some of the cytokines could induce activation of human eosinophils. The inability of these cytokines to activate guinea pig eosinophils must have been due to species variation. The functional studies were all very similar, ie. the amount of H_2O_2 produced, the number of cells adhered and the levels of $[Ca^{2+}]_i$. The drawback with human eosinophil experiments, is the variability of cells numbers obtained, the cost and most importantly the availability of donors.

In conclusion this study has shown that guinea pig eosinophils are a good substitute for human eosinophils as long as care is taken when interpreting the results, eg. the actions of SK&F 96365. More recently the guinea pig chemokine, eotaxin, has been identified by Jose *et al.* Eotaxin, which is generated *in vivo* in a guinea pig model of allergic inflammation, has been found to be a potent eosinophil chemoattractant (289). The cloning by Rothenberg *et al* of the cDNA has revealed high homology with the human cytokines MCP-3, MCP-1 and to a lesser degree MIP-1α and RANTES (290). In our own laboratories guinea pig RANTES has been produced. With the identification of further guinea pig cytokines, the guinea pig eosinophil will prove a useful tool in assessing the functional properties of these cytokines.

7.10 Eosinophilic cell lines - a good substitute?

The matured Eol-1s failed to take on any of the eosinophil characteristics. They did not appear to develop cytoplasmic granules or the nucleus did not become multi-lobed as observed under the microscope. They were very poor producers of H_2O_2 when stimulated by any agonist including PMA. They did elevate $[Ca^{2+}]_i$, but not only

to agonists characteristic of eosinophils. Finally, looking at phosphorylation patterns, upon maturation, they did not appear to resemble the patterns present in human eosinophils. In conclusion, the eosinophilic cell lines were a poor substitute for the primary cells due to the inability to successfully mature the cells. Eols may still prove to be useful, but further studies on maturation are required.

7.11 Implications on future therapeutic strategies in asthma

This work has been carried out to indentify early stages in eosinophil recuitment and/or function which may lead to possible routes of intervention in respiratory disesases including asthma. Bronchodilators are the most commonly used drugs for asthma; they relieve the symptoms generated by histamine and other bronchoconstrictors soon after exposure to an allergen. Historically, xanthines were introduced in the 1930s, corticosteroids in the 1950s, β agonists in the 1960s and sodium cromoglicate in the 1970s. These have been improved over the last two decades to produce the xanthines, glucocorticoids and β_2-agonists used today (291) (see table 7.2).

Table 7.2 Classification and action of anti-asthma drugs. Adapted from *TIPS* 1992. **13**(March), 'Pharmacology of Asthma' poster.

Anti-asthma drug	Classification	Action
β_2-agonists eg. terbutaline (short) salmeterol (long)	Symptomatic	Blocks mast cell activation. Reduces bronchoconstriction & oedema formation
Muscarinic antagonists eg. ipratropium bromide	Symptomatic	Blocks acetycholine, thus reflex action
Xanthines eg. theophylline	Symptomatic/ Prophylactic	Reduces bronchconstriction, oedema formation & leukocyte chemotaxis
Glucocorticoids eg. budesonide	Prophaylactic/ Anti-inflammatory	Reduces oedema, leukocyte chemotaxis, and reduces scar formation from tissue damage. Blocks acute bronchial hyperresponsiveness
Cromoglicate	Prophylactic	Inhibits eosinophil, macrophage and platelet activation. Mast cell stabilisation? Reduces neural stimulation & leukocyte chemotaxis/ activation. Blocks acute bronchial hyperresponsiveness

The present treatments for asthma are highly efficient and improve the quality of life for the patient tremendously, but glucocorticoids and β_2-agonists do not cure asthma. Once treatment has ceased, relapse of the disease often occurs with the same severity of bronchial hyperreactivity and other symptoms. Also, side effects are frequently associated with steroidal treatment. Apart from the symptoms that can be alleviated by the current drugs, bronchial asthma leads to permenant architectural changes in the lung. These changes include subepithelial fibrosis and bronchial smooth muscle thickening and may account for the persistant bronchial hyperresponsiveness

characteristic of the disease. Existing therapies do not reverse these manifestations.

Clearly, PKC and PI 3-kinase are involved in eosinophil function. For these pathways to be used in the development of a therapy, the specific role of PKC and PI 3-kinase in eosinophils needs to be investigated. Different inhibitors of the specific PKC isotypes need to be identified and tested individually to see how these effect eosinophil function. Wortmannin appears to be non-specific at quite low doses (0.05-1μM) and various derivitives based on this compound with more specificity need to be produced.

7.12 Final conclusions

This thesis presents eosinophil functional data, where various agonists implicated in inflammation are able to elicit H_2O_2 production, cause cellular adhesion and $[Ca^{2+}]_i$ elevation from eosinophils. For many of the agonists this study was the first characterisation that they were able to elicit such functional responses in human eosinophils. Different functional states of guinea pig eosinophils also appeared to exist as activity could be up-regulated as demonstrated by GM-CSF and IL-5 or modulated by recruitment into the lung from blood circulation. This data was not obtained for human eosinophils. The effect of recruitment into the lung on human eosinophil function will be very difficult to quantify as many patients will be already undergoing treatment which may adversely effect the results.

This study indicates that the transduction mechanisms for cellular adhesion may lead to the respiratory burst, but the respiratory burst can be activated by an independent mechanism as demonstrated by H_2O_2 production from eosinophils in

suspension. Also, eosinophil adhesion and the respiratory burst were dependent on extracellular Ca^{2+} and Mg^{2+}, whereas $[Ca^{2+}]_i$ elevation was partly dependent on extracellular Ca^{2+} but not Mg^{2+}. Another important result to note is that a rise in $[Ca^{2+}]_i$ elevation was not sufficient to activate the NADPH oxidase complex.

A key role exists for cPKC, and PI3-kinase possibly through aPKC-ζ in H_2O_2 production stimulated by soluble agonists relevant to allergic inflammation. The significant inhibition of H_2O_2 production by either Ro 31-8220/002 or wortmannin suggests these two pathways maybe in some way connected, perhaps different stages in the signalling cascade. Some PTKs may also play a role in O_2^- generation as demonstrated by the PTK inhibitor herbimycin A. This mechanism may be largely redundant due to the presence of other intracellular signalling pathways, but when they are inhibited by either Ro 31-8220/002 or wortmannin, a PTK pathway may account for the residual H_2O_2 production detected.

The cPKC and PI3-kinase pathways are also involved/linked to eosinophil adhesion as the selected inhibitors caused reductions in eosinophil adhesion. This is quite distinct from the PTK pathway, where the tyrosine kinase inhibitors potentiated guinea pig adhesion. A schematic diagram in figure 7.1 shows the possible signalling pathways.

The guinea pig eosinophils were a good model of human eosinophils but with limitations. They exhibited similar functional pathways to human eosinophils with respect to cPKC and PI 3-kinase, however differences occurred between the influx experiments in particular the SK&F 96365 results. In addition there were far fewer agonists that activated guinea pig eosinophils than human eosinophils.

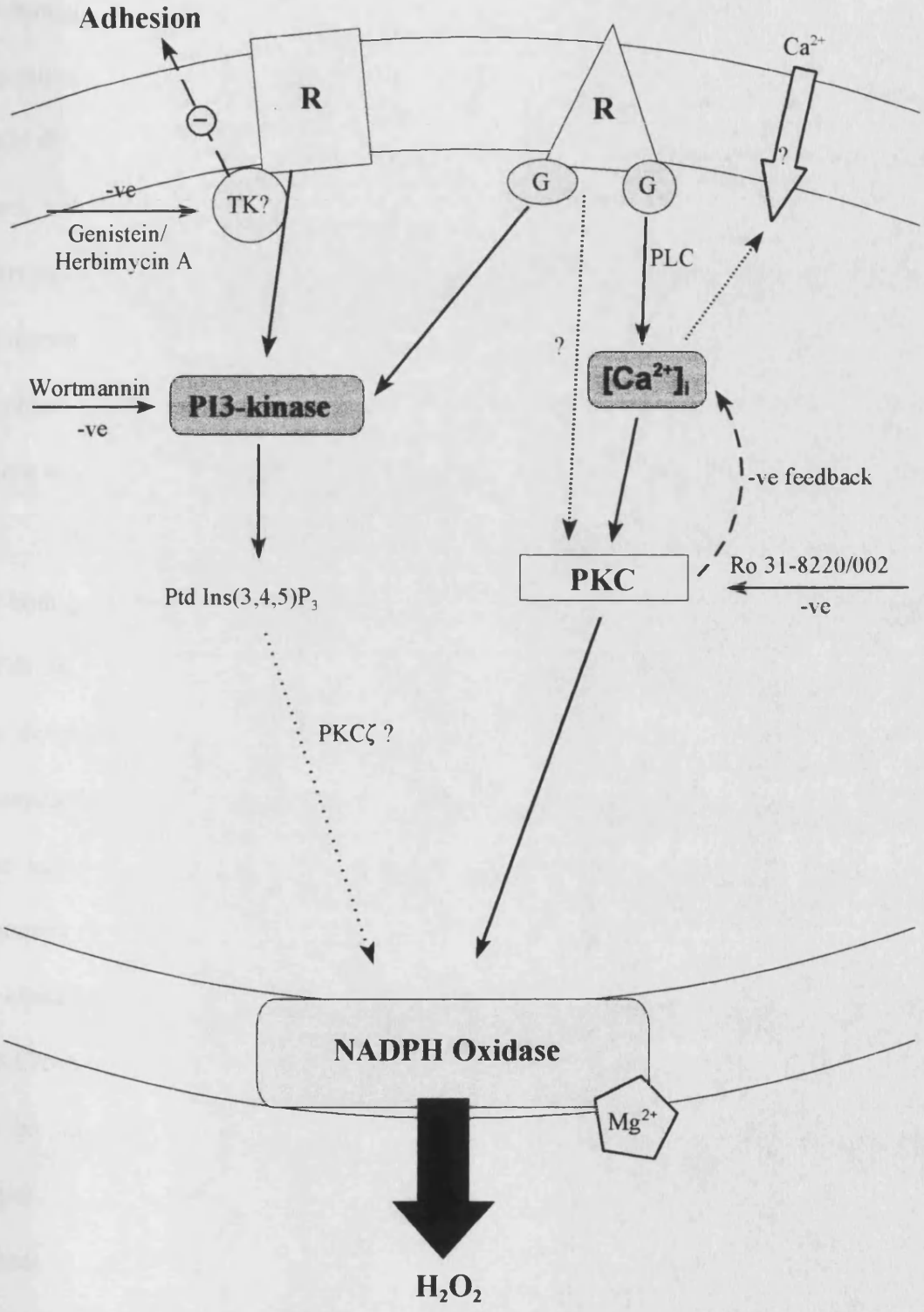


Figure 7.1 Possible signal transduction mechanisms leading to eosinophil activation

This thesis also looked at the cell lines Eol-1 and Eol-3 as viable alternatives to human mature eosinophils for investigation into the functional responses to added agonists. It became apparent that the Eol's were still a long way removed from the fully differentiated cells (even though many maturation processes were tested) and as such were not useful in determining any of the eosinophil functions. This is a particular area of research that should be pursued as one problem with eosinophil research is that only small numbers of primary cells can be obtained, a particular problem for Western blotting experiments. New cell lines should be developed that are more closely related to mature eosinophils.

It is obvious from the experiments presented in this thesis that cellular functions of both guinea pig and human eosinophils can be activated by a wide range of agonists. With the role of the eosinophil implicated in respiratory diseases the strategic approach in developing future therapies would be not to target specific agonists and their receptors as new agonists are constantly being identified, but look at pivotal points in the activation pathway. This research has identified some of the common links between the pathways from receptor activation to functional response eg. PKC and PI 3-kinase, and some distinct pathways of activation eg. requirement for $[Ca^{2+}]_i$ for PKC/NADPH oxidase activation though not essential. The potential target points need to be selected with great care to avoid disruption of similar pathways in other cell types. Also depending of the particular patient requirements, any therapeutic approach needs to be balanced so that the eosinophil does not become completely inoperable. Thus, selective control of different elements in each pathway in conjunction with existing methods for the specific control of airway disease should be developed.

8 References

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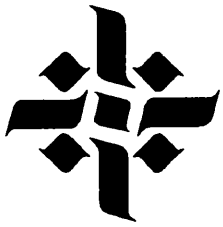

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